Antibody-Redirected T-Cell Immunotherapy for Brain Tumors

by

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Dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
in the Department of Pathology in the Graduate School of
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ABSTRACT

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Abstract

The most common primary malignant brain tumor, glioblastoma, is uniformly fatal. Current therapy provides only incremental benefits in survival and is often incapacitating owing to limits defined by nonspecific toxicity. By contrast, immunotherapy offers a particularly promising approach, and has the theoretical potential to target and eliminate malignant cells with unprecedented specificity. The goal of this dissertation is to apply recombinant technologies to develop a new immune-based therapy for patients with malignant glioma. This work will span the design, production, and preclinical testing of a novel bispecific antibody designed to redirect T cells against a tumor-specific mutant of the epidermal growth factor receptor, EGFRvIII.

Chapters 1 and 2 will provide an overview of broad topics in antitumor immunotherapy and immune biology, with special focus on concepts as they relate to tumors of the central nervous system. In addition, the history and current state of bispecific antibodies, particularly those of the bispecific T-cell engager (BiTE) subclass, as well as their potential role in the treatment of malignant disease, will be considered in detail. Data presented in Chapter 3 will describe our approach to generating novel bispecific tandem single-chain antibody reagents, while experiments in Chapter 4 will demonstrate the capacity of one of these molecules, an EGFRvIII-specific BiTE, to achieve antitumor efficacy both *in vitro* and *in vivo* using murine models of glioma. Addressing a major barrier to the translation of immune therapies for cancer, chapter 5

will establish a potential role for BiTEs in overcoming cell-mediated immune suppression associated with malignant disease. Lastly, Chapter 6 and 7 will report on emerging areas of study, including the use of syngeneic, transgenic murine systems, and strategies by which BiTEs may be propelled rapidly into early phase clinical trials.

In summary, separating BiTEs from other available immunotherapeutic approaches, our work in this field suggests that BiTEs are (1) highly-specific molecules that greatly reduce the risk of toxicity, (2) have the ability to penetrate the blood-brain barrier and accumulate in intracerebral tumors, and (3) may potentially overcome multiple mechanisms of immunosuppression present in patients with glioblastoma. Together, these studies have the potential to improve the clinical management of patients with glioblastoma through the generation of a novel therapeutic.

Dedication

This dissertation is dedicated to all whose lives have been touched by cancer.

Contents

List of Tables

List of Figures

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It has been my great honor to receive support and encouragement from several individuals over the past years, without which this ambitious yet rewarding work could not have been possible. I owe tremendous gratitude to my mentor, Dr. John Sampson, for his deep investment in my education and development as a scientist; Dr. Sampson's many influences as a teacher, friend and role model will certainly continue to enrich my career and provide inspiring benchmarks of success for years to come. I would also like to take this opportunity to acknowledge the members of my dissertation committee, Dr. Darell Bigner, Dr. Duane Mitchell and Dr. Kent Weinhold, whose steadfast, expert counsel provided invaluable guidance as I progressed in my graduate training toward completion. In addition, I offer sincere thanks to Dr. Allan Friedman and Dr. Soman Abraham for their valued professional advice and institutional support from the Division of Neurosurgery and Department of Pathology, respectively. Among others who should not go unnoticed: Dr. Gary Archer, Patrick Gedeon, Dr. James Herndon II, Dr. Chien-Tsun Kuan, Dr. Ira Pastan, Charles Pegram, Dr. Zachary Reitman, Dr. Luis Sanchez-Perez, David Snyder, Scott Szafranski and all colleagues of the Preston Robert Tisch Brain Tumor Center at Duke and Duke Brain Tumor Immunotherapy Program.

Finally, thank you to my family—especially Mom, Dad, Katie and Daniel—and most importantly to my wife, Karmel. Without your love and support I would not be here today. I hope you know that this dissertation belongs to all of you as well. *AMDG.*

1 Overview of Brain Tumor Immunotherapy and Immunobiology

1.1 Permissions and Collaborative Work

This dissertation contains original material as well as excerpts of text and figures from published articles, which were reproduced with permission in accordance with journal and publisher policies as described in Appendix. Although this dissertation is representative of primary work performed by the candidate, assistance was graciously received on several occasions from collaborators listed in Acknowledgements.

1.2 Introduction to Overview

Of all causes of death, cancer is perhaps the most devastating; it currently represents approximately one of every four deaths in the United States and boasts an economic impact outweighing that of any other disease worldwide (Jemal et al., 2010). Relatively little progress has been made in terms of improving prognosis for advanced stage cancers over the past 40 years (Bailar and Gornik, 1997).

Glioblastoma (GBM) is the most common and most aggressive primary malignant brain tumor. Despite numerous advances in therapy—including image-guided surgical resection (Kelly, 1992), high-dose external beam radiotherapy, antiangiogenic treatments (Vredenburgh et al., 2007), and chemotherapy—patients with GBM live less than an average of 15 months from the time of diagnosis (Stupp et al., 2009). Moreover, these treatments are often non-specific and result in incapacitating

damage to surrounding normal brain and systemic tissues. A promising alternative is the use of the immune system, which has the theoretical capacity to eliminate neoplastic cells while leaving healthy cells intact. Although first proposed over a century ago, the concept of antitumor immunotherapy has historically struggled to successfully translate effective treatments for patients with cancer. This changed dramatically in 2010 after pivotal approvals by the Food and Drug Administration for immune-based cancer treatments, sipuleucel-T (Kantoff et al., 2010) and ipilimumab (Hodi et al., 2010), in the treatment of hormone-refractory prostate cancer and metastatic melanoma, respectively. Despite these recent advances, the use of immunotherapy as a standard-of-care treatment for GBM has yet to be realized. A number of promising approaches are currently in development towards this end, and a broad understanding of their applications particularly in the context of intracerebral tumors represents an emerging priority.

Table 1: Historical perspective on progress in therapy for brain tumors.

Year	*Milestone*	*Median Survival*
1887	First successful surgical removal of a brain tumor	6 months
1970s	First promising chemotherapy for glioma (BCNU)	
1975-80	Radiation established as standard treatment for GBM	9 months
1980s	Gamma knife (stereotactic radiosurgery) is introduced	
2003	Chemotherapy carmustine (BCNU) "wafer"	
2005	Oral chemotherapy temozolomide receives approval	14.6 months
2008	Bevacizumab (Avastin) approved for recurrent GBM	

1.3 *Central Nervous System Immune Privilege*

Despite conventional notions of CNS immune privilege, growing evidence supports that both immune cells and antibodies have the capacity to penetrate and treat tumors in the brain. The following section will provide a historical perspective on this subject, as well as summarize more recent findings as they pertain to CNS immune access, particularly in the context of malignancy and antitumor immunotherapy.

1.3.1 Classical Experiments and Emerging Ideology

Antitumor immunotherapies, in the context of intracerebral tumors, encounter a distinct set of challenges, one of the most prominent being that of central nervous system (CNS) immune privilege. The first studies to suggest the concept of limited immune surveillance in the CNS and other select tissues were first reported in 1948 by Sir Peter Medawar, who showed that allogeneic tissue grafts transplanted into the brains of experimental animals were not rejected (Medawar, 1948). Later research in the area of neuro-immunology would support this finding based on unique characteristics that are now generally associated with the CNS: the presence of a specialized blood-brain barrier (BBB) and the absence of conventional draining lymph nodes as well as resident antigen-presenting cells (APCs) within the brain (Hart and Fabre, 1981, Fabry et al., 1994).

While the CNS certainly exhibits immune privilege to some degree, a growing body of data suggests that its isolation from the immune system is not as complete as

once believed. For instance, despite the BBB, immune cells have been shown to traffic to the brain relatively frequently (Owens et al., 1994, Engelhardt and Ransohoff, 2005, Hickey, 1991), and, contrary to what was previously thought, antigen egress *via* cerebrospinal fluid (CSF) compartments and cervical lymphatics also appears to occur (Cserr and Knopf, 1992, Goldmann et al., 2006). Furthermore, it has been proposed that specialized microglia (Gehrmann et al., 1995) along with astrocytes (Aloisi et al., 2000) and certain cells of the choroid plexus epithelium (Serot et al., 1997) are able to mediate human leukocyte antigen (HLA) presentation, thereby functioning as surrogate APCs within the CNS.

1.3.2 Crossing the Blood-Brain Barrier

As previously mentioned, immune cells, specifically activated T lymphocytes, have the ability to penetrate the BBB under normal physiological conditions. This was first appreciated when experimental animals were injected intravenously with radioactively labeled T cell blasts, which were subsequently tracked to the CNS (Engelhardt and Ransohoff, 2005). Naïve T lymphocytes, however, are significantly restricted from entering the CNS, suggesting that penetration past the BBB is possible only after activation takes place (Mitchell et al., 2008a). When it does occur, lymphocyte extravasation into the brain parenchyma is a highly-regulated process mediated by several well-characterized adhesion molecules and chemotactic factors (Engelhardt and Ransohoff, 2005). Once inside the CNS compartment, whether T lymphocytes

proliferate and differentiate within the brain microenvironment has yet to be established, as previous studies differ on this point; nevertheless, it has been shown that these cells do remain in the CNS for longer periods of time if given the opportunity to interact with their cognate antigen (Deshpande et al., 2000, Masson et al., 2007).

Central-memory T lymphocytes that alternatively enter the CNS *via* the choroid plexus (Ransohoff et al., 2003) flux continuously throughout subarachnoid spaces, and have purportedly significant roles in routine CNS immunosurveillance. Subarachnoid-space macrophages and pericytes associated with CNS microvasculature are both considered to be critical in the presentation of recall antigens to this T cell population. At any given time, T lymphocytes represent over 80% of the approximately 150,000 cells normally found in the CSF of healthy individuals (Engelhardt and Ransohoff, 2005). As an absolute number, this somewhat diminutive quantity of cells may not be particularly relevant to immune responses within the brain parenchyma; however, it seems that these cells are relatively CSF-enriched, given that lymphocytes typically compose less than 5% of all leukocytes present in circulating blood.

In its intact state, the BBB is thought to be poorly permeable to antibodies. This assumption stems from the observation that CSF titers in normal individuals are relatively low, especially in comparison to those measured in peripheral blood. Generally, the rate of immunoglobulin diffusion into the CNS varies depending on the molecular weight of a given protein (Pardridge, 1991); as an example, the physiological

CSF/serum ratios for IgM and IgG have been quoted to range from 0.005% to 0.025% and 0.16% to 0.32%, respectively, reflecting the difference in size between these molecules (Bickel, 1995). Although these limited ratios undoubtedly evidence CNS immune privilege to some degree, classic animal experiments have verified that, after both active and passive immunizations, corresponding antibodies can be detected within the CNS, specifically the brain, spinal cord, and CSF (Freund, 1930). However, the reported fraction eventually isolated from these areas was again notably small—0.1 to 1% of that found in serum.

The theoretical possibility that even small amounts of antibody can cross the BBB and have physiologically relevant effector functions in the CNS is supported by the recent development of promising vaccines for patients with Alzheimer's disease (AD). These vaccines target amyloid-β (AB), the cleavage product of amyloid precursor protein (APP), mutations in which have been shown to lead to parenchymal amyloid plaque accumulation (Hardy, 1997, Selkoe, 1999) in addition to other pathological features and clinical manifestations of AD (Lue et al., 1999, Lahiri et al., 2005). Initial experiments using transgenic mice expressing mutant APP have shown that active immunization with the AB peptide reduces plaque burden and improves behavioral endpoints (Koller et al., 2004, Brendza and Holtzman, 2006, Schenk et al., 1999). This provided the first evidence that an immune response can be used as a potential treatment for AD, in theory by preventing formation of amyloid deposits and mediating

clearance of preexisting plaques. Subsequent studies confirmed that the therapeutic effects of the vaccine are, at least in part, due to an antibody-mediated mechanism. This was primarily demonstrated by animal experiments showing that peripherally administered AB-specific antibody enters the CNS, localizes to plaques, and achieves amyloid clearance mimicking that observed in previous mouse studies employing active immunization strategies (Bard et al., 2000, Bacskai et al., 2001). Several hypotheses have been offered regarding the mechanism behind antibody-mediated plaque clearance in AD (St George-Hyslop and Westaway, 1999, Duff, 1999). Of these, one prominent theory states that passively administered antibody sequesters AB-peptide in the periphery without crossing the BBB, thereby generating a concentration gradient favoring efflux out of the brain (DeMattos et al., 2001). Other suggested antibody mechanisms rely on passage of antibody across the BBB; these include direct plaque disaggregation (Solomon et al., 1997) and Fc receptor-mediated microglial phagocytosis (Bard et al., 2000).

Given these conclusions, the concept of BBB permeability in the absence of frank inflammation appears to be garnering support. However, it has long been asserted and widely-accepted that in the presence of neuroinflammatory disease states—including experimental autoimmune encephalitis (EAE), meningitis, and cancer—the BBB undergoes changes that alter its ability to block the migration of leukocytes and serum proteins into the CNS (de Vries et al., 1997). Furthermore, by virtue of their existence,

paraneoplastic syndromes clearly demonstrate that such changes in the BBB occur, and that these changes are in fact clinically significant. Most paraneoplastic neurological disorders (PND) are likely immune mediated (Dalmau and Rosenfeld, 2008), as suggested by the demonstration of antineural antibodies in the CNS of patients with peripheral tumors. These antibodies represent the body's natural immune reactivity against systemic tumor antigens, and cross-reactivity with neurological structures has been found to result in significant morbidity. A number of paraneoplastic antibodies have been cited in the involvement of PND pathogenesis; these include anti-Hu, anti-Yo, anti-Ri, anti-CV2/CRMP5, anti-Ma, and anti-amphiphysin antibodies (Dalmau and Rosenfeld, 2008).

Since pathological antibodies have been shown to cross the BBB in the context of malignancy, it follows that peripherally administered therapeutic antibodies should also have access to the intracerebral environment with physiologically relevant outcomes. The development of radio-labeled monoclonal antibodies for the diagnosis and treatment of brain tumors was first explored by Day and coworkers in 1965 (Day et al., 1965), and since then, numerous studies have supported that monoclonal antibodies (mAbs) are capable of localizing to intracerebral malignancies. Using radioiodinated anti-tenascin mAb 81C6, our group has shown that not only does 81C6 exhibit therapeutic activity in mice with intracranial human glioma xenografts, but that selective tumor localization also occurs in patients with a variety of intracranial

malignancies following peripheral administration of the antibody (Zalutsky et al., 1989). However, tumor-specific uptake of 81C6 remained quite low at less than 5×10^{-3} % of the injected dose per gram, and nonspecific antibody accumulation also took place in other tissues besides the brain including the liver, spleen and bone marrow. In contrast to what was observed with 81C6, results from later human studies using radiolabeled chimeric ch806, a mAb specific for the mutated epidermal growth factor receptor, EGFRvIII, suggest that higher-percentage BBB penetration may be achieved in the absence of cross-reactivity with systemic antigens, effectively creating an intracerebral antigen sink at the tumor site (Scott et al., 2007a). Using single-photon emission computed tomography (SPECT), this potential effect was observed given the physiological localization of Indium-111-labeled ch806, which was noted to accumulate within intracranial target lesions without visual evidence of nonspecific, residual binding in normal tissues.

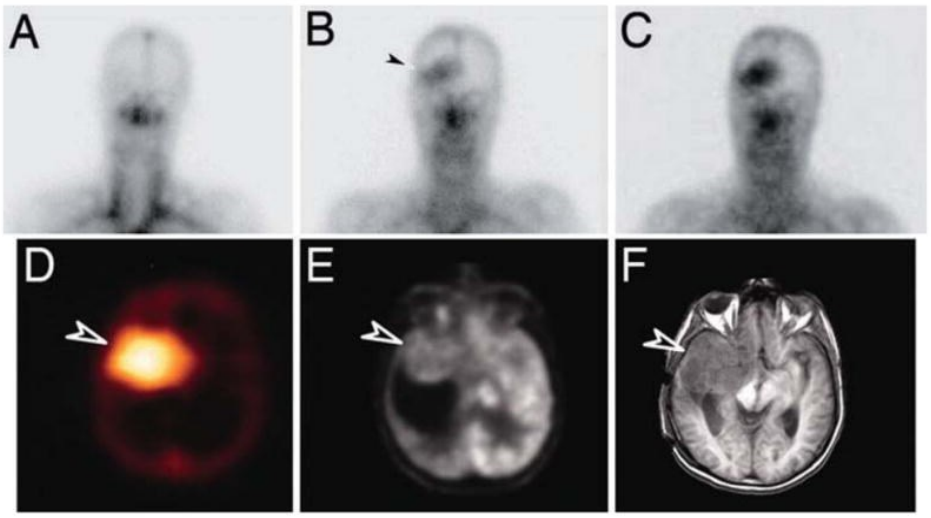


Figure 1: Targeting of glioma by radiolabeled chimeric monoclonal antibody directed against the EGFRvIIII tumor antigen.
(*A*–C) Planar images of the head and neck obtained on day 0 (*A*), day 3 (*B*), and day 7 (*C*) after infusion of ^{111}In-ch806. Initial blood pool activity is seen on day 0, and uptake of ^{111}In-ch806 in an anaplastic astrocytoma in the right frontal lobe is evident by day 3 (arrow) and increases by day 7. (*D*–*F*) Tumor-specific uptake of ^{111}In-ch806 (arrow) is demonstrated in a SPECT image of the brain (*D*), ^{18}F-FDG (FDG, Fluorodeoxyglucose) positron emission tomography (*E*), and MRI (*F*). Figure reproduced according to journal guidelines (Scott et al., 2007a).

1.4 Tumor-Associated Rejection Antigens

The landmark paper published over two decades ago by van Pel and Boon rekindled the then waning interest in cancer vaccine development when it suggested that even non-immunogenic tumors display sufficiently "foreign," and therefore immunologically susceptible, antigen profiles (Van Pel and Boon, 1982). Since that time, a great deal of effort has gone towards characterizing a variety of human tumor antigens, the majority of which can now be placed into one of two main categories:

those consisting of over-expressed normal gene products or, alternatively, those derived from mutations in somatic genes (Gilboa, 2004, Gilboa, 1999).

Most well-characterized targeted tumor antigens isolated to date correspond to over-expressed proteins that are also present in normal cells, two examples of which include CD20 and erbB2, proteins associated with lymphoma and breast cancer, respectively. The ability of antigens in this category to mediate optimal tumor rejection, however, is often compromised by the fact that proteins that are also found on normal cells have the potential to trigger immunologic tolerance to varying degrees. Notable exceptions to this limitation include antigens associated with fetal gene products, such as carcinoembryonic antigen (Kass et al., 1999), or those expressed solely in immunoprivileged, tissue-specific sites like the testis. The latter group includes the melanoma MAGE, GAGE (Van den Eynde et al., 1995), and BAGE (Boel et al., 1995) family antigens, all of which, due to their limited expression, trigger little to no tolerance and should therefore make ideal tumor rejection antigens.

Cancer vaccination protocols that effectively target normal gene products invariably pose the risk of autoimmune toxicity (Gilboa, 2004). This untoward effect can be avoided to some extent by directing the immune response against a mutated protein specific only to tumor cells. As targets, these antigens have the advantage of avoiding central tolerance mechanisms, in theory making them more suitable for tumor rejection. However, a limitation of these antigens is that they are generally patient-specific as they

often reflect random mutations associated with the inherent genetic instability of tumors (Lengauer et al., 1998, Loeb, 2001). Thus, to the extent that mutated gene products are incidental to the oncogenic process, they are conceivably restricted in their use as practical targets of widely applicable cancer vaccines. Conversely, although the majority of somatic mutations in tumors does appear to be sporadic (Lennerz et al., 2005), recent studies using high-throughput screening have suggested that several functional mutations associated with, rather than incidental to, the oncogenic process are in fact not random, and that these variants are consistently shared among patients (Thomas et al., 2007). The challenge then, is to isolate and target these ideal antigens: frequent, highly specific, oncogenic mutations that are also absent from normal tissues, thereby avoiding the risk of autoimmunity. To date, few such antigens are known, though their discovery represents a potential boon for the further development of effective antitumor immunotherapies.

1.5 Glioma-Induced Immune Suppression

Gliomas are also known to potentiate a hostile environment that can antagonize the development of efficacious antitumor immune responses. Immune suppression in patients with GBM is manifested by several metrics including low peripheral lymphocyte counts, depressed skin reactivity to recall antigens, and impaired T-cell function with a counterproductive Th2 cytokine skew. A number of pathways have been shown to contribute to the development of this pro-tumorigenic milieu. Like other

cancers, gliomas secrete immunosuppressive cytokines including prostaglandin E2 (PGE-2), transforming growth factor (TGF)-ß and interleukin (IL)-10, leading to potentiation of immune resistance through down-regulation of major histocompatibility complex (MHC) expression and suppression of both normal T-cell proliferation and dendritic cell (DC) maturation (Heimberger and Sampson, 2011). In addition to soluble secreted factors, specific subsets of immune regulatory cells have also been cited as central mediators of tumor-associated immune suppression. Among these include tumor-associated myeloid-derived cells and macrophages (Pollard, 2004), as well as a subset of T cells known as regulatory T cells (T_{regs}), which have perhaps been most frequently implicated. T_{regs} have not only been shown to be present at increased proportions in both the peripheral blood and tumors of patients with GBM (Fecci et al., 2006, El Andaloussi and Lesniak, 2006), but their presence has also been correlated with the overall malignant behavior of these tumors (Heimberger et al., 2009). T_{regs} are known to suppress effector T-cell responses, specifically by restricting IL-2 production and inducing T-cell anergy. Importantly, T_{reg} depletion has been positively associated with enhanced immunity in patients with GBM (Sampson et al., 2012), and *in vivo* impairment in tumor-bearing mice has been shown to prolong survival (El Andaloussi et al., 2006).

Gliomas not only possess low levels of surface MHC, but are also known to express costimulatory inhibitory molecules which may lead to direct inhibition of immune responses (Parsa et al., 2007). These interactions may be manipulated by GBM

cells to unfavorably alter signaling through immune checkpoints such as programmed cell death (PD)-1 and cytotoxic T-lymphocyte antigen (CTLA)-4. Other areas of investigation have underscored the role of signal transducer and activator of transcription (STAT)3 in regulating inflammatory responses. Interestingly, the STAT3 pathway in both immune cells (Kortylewski et al., 2005) and tumor cells (Wei et al., 2010) has been shown to potentiate immune suppression, leading to generalized impairment of effector responses. As such, therapies designed to modulate STAT3 as well as costimulatory signaling (Fecci et al., 2007) to enhance antitumor immune responses are among many promising therapies for GBM under current investigation.

1.6 Clinical Applications of Glioma Vaccines and Immune-Based Approaches

The overall goal of tumor immunotherapy is to develop or enhance immune responses against established disease, even in cases where natural immune surveillance has either failed or been rendered less effective by mechanisms of tumor-associated immune evasion. To date, a wide range of immune-based strategies have been developed towards this end. Among these include passive infusion with antibodies as well as adoptive transfer of tumor-reactive lymphocytes, cytokine-based stimulatory treatments, and an array of active immunization methods in the form of vaccines administered to elicit host immunity against tumor cells. The following sections will

introduce the basic principles for several of these modalities, with a focus on major advances that have emerged from representative clinical trials.

1.6.1 Active Immunotherapy for Brain Tumors

Active immunotherapies seek to achieve priming of host immunity and immunological memory against tumors through the use of vaccines. Over the years, approaches to establishing active immunotherapy against brain tumors have varied widely, and in some cases have led to successful advancement toward phase III randomized studies.

1.6.1.1 Autologous Tumor Cell Vaccines

One of the oldest methods of active immunization for cancer consists of direct vaccination with tumor cells, which in some cases have been modified to increase immunogenicity. In this approach, autologous tumor cells are resected during surgery and, following inactivation by radiation, are incorporated into a vaccine to elicit immune reactivity in the individual from whom the tumor was isolated. Early clinical studies have been performed with tumor cells that have been genetically modified to enhance biological activity, either through inhibition of immunosuppressive factors (*e.g.*, TGF-ß) (Fakhrai et al., 2006), or increased secretion of immune potentiating cytokines including IL-4 (Okada et al., 2000), IL-12 (Ehtesham et al., 2002) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Overall, approaches using autologous tumor cell

vaccines have been shown to elicit antitumor immune responses and to be well-tolerated without severe adverse events.

Compared to other methods, vaccination with whole tumor material has a distinct advantage of eliciting immune responses that are tailored to each individual tumor, thereby accounting for the broad interpatient variability and heterogeneous antigen expression associated with gliomas. However, for the same reasons, whole tumor vaccines are conversely hampered by a certain lack of methodological standardization across trials and also in a technical sense owing to the need for a significant amount of tumor tissue. Other disadvantages of using whole tumor cells include the theoretical dilution of tumor antigens by normal cellular components, and difficulties associated with isolating and characterizing antigen-specific immune responses following vaccination.

1.6.1.2 Peptide Vaccines

Peptide vaccines comprise delivery of a tumor antigen, often in conjunction with an immune-stimulating adjuvant. Peptide vaccines represent an especially attractive platform for active immunotherapy, given that they are relatively easy to manufacture, can be tailored to individual or mixed antigens, and can be standardized in formulations across multiple institutions. Several groups have demonstrated that peptide vaccines can be administered in the setting of malignant glioma to achieve tumor-specific immune responses in clinical trials. One notable peptide vaccine, the EGFRvIII-specific

PEP-3-KLH (CDX-110) formulation, has been shown to elicit EGFRvIII-specific immune responses, leading to subsequent elimination of EGFRvIII-expressing tumor cells in mice and humans (Sampson et al., Sampson et al., 2009, Heimberger et al., 2003). Although Phase 2 clinical studies of peptide vaccines against EGFRvIII and Wilms tumor 1 (WT1) (Izumoto et al., 2008) have yielded encouraging results, one concern is that targeting single antigens may inadequately address the diverse and heterogeneous nature of malignant gliomas, leading to eventual outgrowth of antigen-negative recurrence and immune escape. As such, personalized vaccines in the form of several peptide combinations have also been attempted, which have also demonstrated the capacity to develop specific immunity and favorable responses in clinical trials (Yajima et al., 2005).

1.6.1.3 Dendritic Cell Vaccines

Dendritic cells (DC) are "professional" antigen-presenting cells whose main function is to modulate immune responses through the processing and presenting of antigenic material to effector T cells. Given recent advances in their *ex vivo* generation and manipulation, DCs have emerged as a vaccine platform with great potential for patients with GBM. To manufacture a DC vaccine, autologous DCs are harvested from peripheral blood or bone marrow and exposed *in vitro* to tumor antigens in the form of tumor homogenate, peptides, or even genetic material encoding antigens of interest, prior to injection back into the patient. Several clinical trials have demonstrated that DCs loaded with tumor homogenate or lysate are well-tolerated (Wheeler et al., 2008, De

Vleeschouwer et al., 2008), and may be complemented by certain adjuvant therapies (*e.g.*, Toll-like receptor agonists) (Prins et al., 2011) to produce antitumor T-cell responsiveness. In addition, recent efforts to integrate tumor-pulsed DC vaccines with measures of standard postoperative care have met with early success (Ardon et al., 2010).

Instead of whole tumor lysate, peptides have also been used as an alternative source of antigenic material for the DC vaccine platform. One notable peptide-based DC vaccine targeting EGFRvIII has successfully demonstrated the capacity to elicit EGFRvIII-specific immune responses without serious adverse events (Sampson et al., 2009). Other studies of peptide-pulsed DC vaccines include DCs loaded with multi-epitope antigen formulations either through the use of acid-eluted, tumor-associated MHC class I peptides (Yu et al., 2001, Liau et al., 2005) or synthetic peptide combinations—recent examples of the latter have targeted glioma-associated antigens including HER2, TRP-2, EphA2, IL-1Rα2, AIM-2 YKL-40 and gp100 (Okada et al., 2011, Phuphanich et al., 2013). Lastly, efforts have also been developed to elicit antitumor immune responses using DCs loaded with autologous, tumor-antigen encoding RNA; early clinical trials using this strategy have demonstrated safety and feasibility, particularly in the setting of pediatric brain cancers (Caruso et al., 2004, Caruso et al., 2005).

1.6.2 Adoptive Immunotherapy for Brain Tumors

In general, adoptive immunotherapy refers to the *ex vivo* activation and expansion of immune cells which are then administered to treat patients through intratumoral or intravenous infusion. To date, the most encouraging results for CNS tumors have been achieved in the setting of melanoma brain metastases, where adoptive transfer of either autologous tumor-infiltrating lymphocytes (TIL) or T-cells genetically modified to target tumor antigens resulted in complete response rates of 41% and 22%, respectively (Hong et al., 2010). Despite this promise, adoptive transfer therapies for high-grade glioma have not yet realized this level of success. Historically, clinical trials of adoptive immunotherapy for glioma have attempted transfer of various cell types including lymphokine-activated killer (LAK) (Jacobs et al., 1986) cells as well as cytotoxic T lymphocytes (CTLs) that have been isolated from expanded tumor-infiltrating lymphocytes (TIL) (Quattrocchi et al., 1999) or lymph nodes from vaccinated patients (Plautz et al., 2000). Although several groups have demonstrated the general feasibility of these approaches, therapeutic outcomes have been relatively modest, especially when taking into account the high levels of antitumor cytotoxicity observed during corresponding preclinical studies *in vitro*. As such, current efforts seek to elucidate additional factors that may refine the translation of adoptive transfer therapies *in vivo*; among these include the identification of specific effector cell phenotypes predictive of antitumor responses, as well as improvements to enhance persistence of

infused cells, either through host-conditioning lymphodepletive regimens or the concomitant use of homeostatic cytokines (*e.g.*, IL-7, IL-15).

Table 2: Data from representative immunotherapy clinical trials for glioma.

Agent delivered	Phase	Institution	Median Survival
PEP-3-KLH + GM-CSF (ACTIVATE)	II	Duke University Medical Center/The University of Texas MD Anderson Cancer Center/Celldex	2.4 years; newly diagnosed; *n* = 23.
PEP-3-KLH + GM-CSF with TMZ (ACT II)	II	Duke University Medical Center/The University of Texas MD Anderson Cancer Center/Pfizer	1.9 years; newly diagnosed; *n* = 21.
DCs + PEP-3-KLH	I/II	Duke University Medical Center	1.8 years; newly diagnosed; *n* = 14.
Personalized peptide vaccines (4)	I	Nigata University	1.7 years; recurrent GBM; *n* = 17.
DCs + CMV	II	Duke University Medical Center	not reached but will exceed 1.6 years; newly diagnosed GBM; *n* = 13.
DCs + autologous tumor lysate	II	University of Leuven and Wurzburg	0.8 years from relapse; recurrent GBM; *n* = 56.
DCs + acid=eluted tumor peptides	I/II	Cedars Sinai Medical Center	1.3 years; newly diagnosed GBM; *n* = 7.
DCs + tumor homogenate	II	Cedars Sinai Medical Center	1.8 years; newly diagnosed GBM; *n* = 11. 1.6 years; recurrent GBM; *n* = 21.
DCs + acid-eluted tumor peptides	I	UCLA	2.0 years; newly diagnosed and recurrent GBM; *n* = 12.
Autologous tumor cells with antisense TGF-β	I	Advanced Biotherapies/NovaRx	1.4 years; *n* = 6.
Poly-ICLC	II	North American Brain Tumor Coalition	1.4 years; newly diagnosed GBM; *n* = 30.
Autologous whole tumor with GM-CSF and adoptive transfer of CD3-activated cells	II	Tvax Biomedical	1.0 year; recurrent malignant glioma; *n* = 19.

Table adapted with permission (Heimberger and Sampson, 2011).

1.7 Experimental Allergic Encephalomyelitis

It is likely that as immunotherapies become more potent, the risk of adverse immune side effects may increase. This is especially true for immunotherapeutic platforms designed to target glioma-associated antigens that are shared with normal, healthy brain tissue; active immunization against these antigens could escalate the risk of uncontrolled CNS autoimmunity, similar to what has been observed in preclinical models of experimental allergic encephalomyelitis (EAE). Importantly, following vaccination with tissue derived from human gliomas, lethal EAE or EAE-like toxicity has been documented in both non-human primates (Graf et al., 2002) as well as in human studies of immunotherapy for brain tumors (Bloom et al., 1973, Trouillas, 1973). These results are to some degree expected, given that gliomas are known to express both normal adult and fetal brain antigens, and because several preclinical protocols are already known to produce EAE upon immunization with CNS tissue.

As such, the risk of autoimmune toxicity represents an ongoing concern for tumor vaccines that are not carefully selected for tumor-specificity. However, as mentioned above, this issue can be avoided to some extent by redirecting immune responses against antigens that are expressed solely in gliomas and completely absent from all normal tissues. To date, only few such antigens have been thoroughly characterized. Among these include the tumor-specific mutant protein, EGFRvIII (Choi et al., 2009), as well as frequent and homogenous mutations associated with isocitrate

dehydrogenase 1 (IDH1) (Yan et al., 2009). Also notable is the selective detection of viral antigens corresponding to human cytomegalovirus infection, which have been shown to be expressed in a high percentage of GBM tumors but not surrounding normal brain (Mitchell et al., 2008b, Cobbs et al., 2002).

1.8 The EGFRvIII Tumor-Specific Antigen

Among the many antigens that have been shown to be overexpressed on tumor cells, the type I epidermal growth factor receptor (EGFR) represents one of the most frequently implicated cell-surface markers for a wide range of human malignancies. Functionally, the EGFR has well-characterized roles in oncogenesis and tumor progression, and as such, amplification and overexpression of the *EGFR* gene is considered a poor prognostic indicator (Neal et al., 1990). Regarding intracerebral cancers in particular, the *EGFR* gene is amplified in up to 50% and overexpressed in over 90% of GBM specimens (Ekstrand et al., 1991, Jaros et al., 1992), suggesting significantly augmented cellular activity of this receptor in these tumors.

The EGFR is a 170-kDa transmembrane glycoprotein, consisting of an extracellular ligand-binding domain and an intracellular region with tyrosine kinase functionality (Stoscheck and King, 1986). Activation via stimulatory interactions with growth factors—including epidermal growth factor (EGF) and transforming growth factor-α—results in receptor dimerization and subsequent intracellular autophosphorylation on tyrosine residues, in turn leading to the activation of

downstream molecules associated with cellular mitogenesis and survival (Carpenter and Cohen, 1990). Given the nature of these potentially oncogenic pathways, it was originally believed that the impact of EGFR on neoplastic processes was exclusively due to amplification of its corresponding gene. However, it is now clear that many tumors, including GBM, also express rearranged, aberrant forms of the *EGFR* gene that have significant physiological relevance (Ekstrand et al., 1991, Frederick et al., 2000). Several of these mutations have been reported in the literature and are typically associated with tumors that also exhibit extensive wild-type gene amplification (Libermann et al., 1985, Wong et al., 1992).

The most common and well-characterized *EGFR* mutant was first identified in primary human GBM tumors and is commonly referred to as the EGFR class III variant (EGFRvIII). EGFRvIII is a constitutively active, ligand-independent form of the EGF wild-type receptor (Hou et al., 1997, Batra et al., 1995), the expression of which has been shown to have tumorigenic effects, both augmenting proliferation and inhibiting apoptosis (Nishikawa et al., 1994, Batra et al., 1995). Specifically, EGFRvIII has also been shown to promote greater cellular motility (Pedersen et al., 2004, Boockvar et al., 2003) as well as resistance to radiation and chemotherapy (Lammering et al., 2004b, Lammering et al., 2004a, Montgomery et al., 2000), characteristics often associated with highly malignant tumors.

A number of molecular mechanisms have been implicated in the oncogenic pathways coupled with EGFRvIII downstream signaling. In the absence of ligand-binding and dimerization, for example, EGFRvIII has been observed to constitutively interact with adaptor proteins central to the Ras cascade (Prigent et al., 1996, Chu et al., 1997). Similarly, growth advantage in cells expressing EGFRvIII has been attributed at least in part to elevated phosphatidylinositol (PI) 3-kinase levels and consequent activation of the c-Jun N-terminal kinase (JNK) pathway (Antonyak et al., 1998, Moscatello et al., 1998). The respective involvement of, and interplay among, these signals in neoplastic processes have yet to be fully-described; however, it has been shown that malignant cells become dependent on these pathways to some extent, and that removal of such stimulation results in reduced cell survival (Weinstein, 2002).

Structurally, EGFRvIII is an 801 base pair in-frame deletion of the wild-type receptor that corresponds to mRNA exons 2-7, the absence of which leads to the translation of a truncated extracellular domain. A consequence of this deletion-mutation is the fusion of two otherwise distant portions of the molecule, which in turn creates an antigenic junction characterized by a novel glycine residue, flanked by amino acid sequences that are not typically adjacent in the wild-type receptor (Bigner et al., 1990, Libermann et al., 1985). This tumor-specific epitope has been shown to be present on the surface tumor cells, yet completely absent from any normal adult tissues (Humphrey et al., 1990).

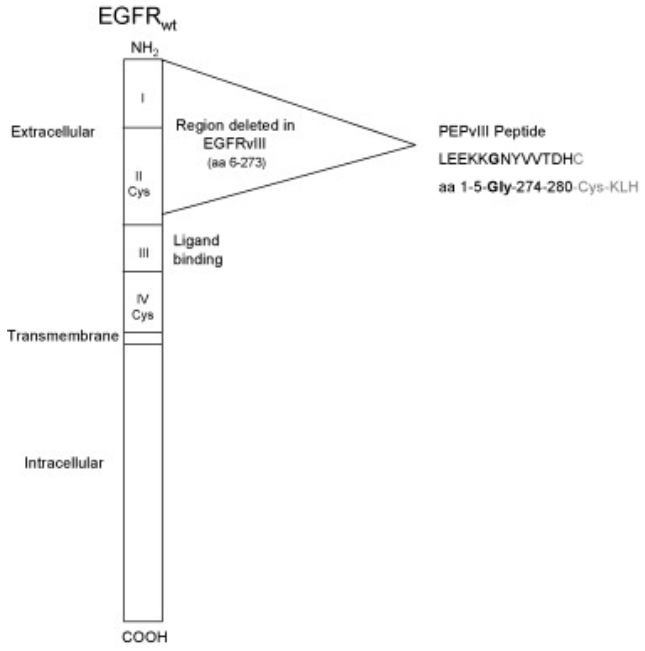


Figure 2: Schematic diagram of the EGFR wild-type protein and EGFRvIII in-frame deletion.

During the deletion amino acids 6 and 273 are split forming a novel glycine at the junction of amino acids 5 and 274. PEPvIII is a 13 amino acid peptide with a terminal cysteine added to facilitate conjugation to KLH. Figure reproduced with permission according to journal policies (Sampson et al., 2008)

Immunohistochemical (IHC) analysis represents one of the most common assays used to identify the EGFRvIII mutant along with a number of second messenger molecules also expressed in malignant cells. Alternative approaches to IHC which employ molecular techniques such as Western blotting and reverse transcription-polymerase chain reaction assays (RT-PCR) are currently being explored and have confirmed the specific expression of EGFRvIII in human GBM specimens; to date, data derived from IHC studies have been shown to be consistent with results obtained using other methods (Feldkamp et al., 1999). As evidenced by IHC, EGFRvIII is consistently

expressed in a wide variety of cancers, and can be found in approximately 20% of GBM specimens (Moscatello et al., 1995). Within such tumor samples, the proportion of EGFRvIII-expressing cells has been shown to range from 37-86% (Wikstrand et al., 1997), suggesting that cells within EGFRvIII-positive tumors may translate the variant receptor with at least some level of homogeneity. Thus, given its oncogenic properties, inherent tumor specificity, and frequent expression in malignancy, the EGFRvIII mutation represents a particularly attractive tumor-specific target for the development of anti-cancer immunotherapies (Kuan et al., 2001, Wikstrand et al., 1998).

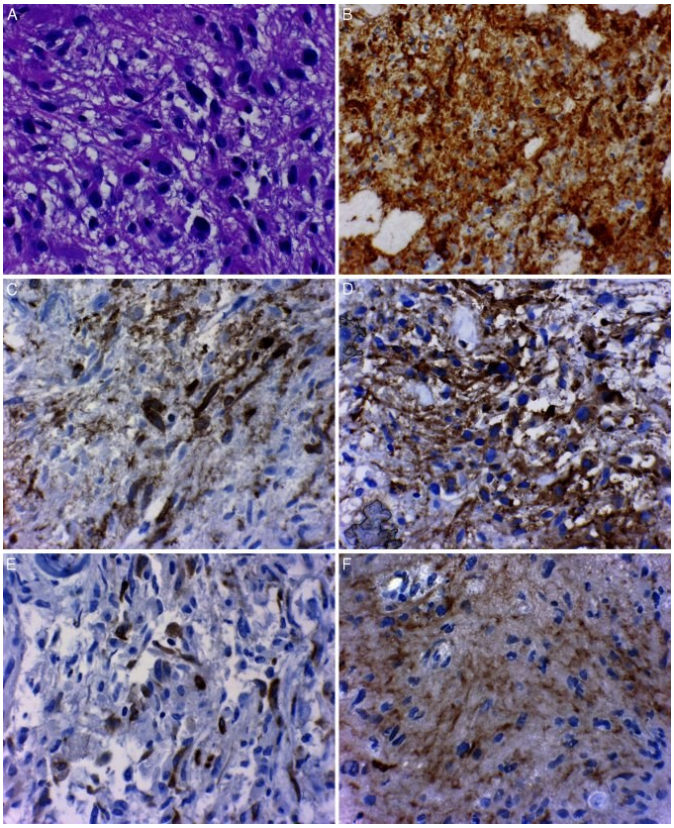


Figure 3: Immunohistochemical analysis of EGFRvIII and other tumor associated messenger proteins in human astrocytoma.

A, High-grade astrocytoma used in subsequent immunohistochemical assays (hematoxylin-eosin, original magnification ×400). B, Anti– epidermal growth factor receptor (anti-EGFR) wild-type immunohistochemistry showing strong diffuse cytoplasmic immunoreactivity, a pattern that is frequently associated with *EGFR* genetic amplification (original magnification ×400). C, Anti-EGFRvIII immunohistochemical reactivity exhibiting strong cytoplasmic localization. EGFRvIII immunoreactivity is most commonly encountered in tumors also exhibiting amplification of the *EGFR* locus (original magnification ×400). D, Anti-phosphatase and tensin homolog (anti-PTEN) immunoreactivity demonstrating 80% of tumor cells with cytoplasmic reactivity, a pattern associated with an intact PTEN status in the tumor (original magnification ×400). E, Anti–phospho-S6 immunohistochemistry revealing approximately 20% of tumor cells labeling, indicating this messenger is activated in this tumor (original magnification ×400). F, Anti–phospho-Akt immunohistochemistry demonstrating approximately 80% of tumor cells labeling, indicating this messenger is activated in this tumor (original magnification ×400). Figure reproduced with permission according to journal policy (McLendon et al., 2007)

1.8.1 Therapeutic Targeting of EGFRvIII

Currently, a number of immunotherapeutic approaches targeting the unique EGFRvIII antigen are under investigation. Although these were mentioned briefly in previous sections covering glioma vaccines and immune-based treatments, the remainder of this chapter will revisit therapeutic targeting of EGFRvIII in detail, with special attention to published studies regarding preclinical development as well as recent advances made toward translation in human trials.

Given the technical difficulty and relatively high cost of dendritic cell (DC) vaccination therapy, the most promising and practical active vaccination format to date is a peptide derived from the novel fusion junction amino acid sequence. PEPvIII (H-Leu-Glu-Glu-Lys-Lys-Gln-Asn-Tyr-Val-Val-Thr-Asp-His-Cys-OH) (Moscatello et al., 1997) is a well-characterized, EGFRvIII-specific, 14-mer peptide that has been shown, when coupled to keyhole limpet hemocyanin (KLH), to elicit both humoral and cellular immune responses. Extensive experience has been reported with PEPvIII-KLH, which has clearly demonstrated the induction of considerable EGFRvIII-specific immune responses in both murine tumor models and early clinical trials.

1.8.1.1 EGFRvIII: Preclinical Studies

Passive administration of EGFRvIII-specific antibodies Y10 and L8A4 (unarmed murine IgG_{2a} and IgG_1, respectively) has been shown to lead to significant tumor growth inhibition in subcutaneous (s.c.) murine melanoma models. These studies, which use

syngeneic tumors transfected with a murine homologue of the variant receptor (msEGFRvIII), have shown that while these two monoclonal antibodies achieve therapeutic efficacy when given intraperitoneally, only those mice treated with Y10 exhibit lasting tumor-free survival after treatment is discontinued (Sampson et al., 2000). Evidence from *in vitro* studies suggests that Y10 has the ability to mediate a wide range of effector functions when incubated with cells expressing msEGFRvIII. These functions include the inhibition of DNA synthesis and cellular proliferation, as well as the activation of autologous, complement-mediated, and antibody-dependent cell-mediated cytotoxicity (ADCC).

Active vaccination strategies targeted against msEGFRvIII in syngeneic murine tumor models have also proven to be effective. Following intraperitoneal injection with DCs pulsed with PEPvIII-KLH, C3H mice that had previously been challenged with intracerebral tumors demonstrated a significant increase in median survival. Furthermore, all the mice in this study survived rechallenge with tumor, suggesting that immunization was sufficient to create long-term immunological memory against the msEGFRvIII antigen in this model system (Heimberger et al., 2002). Following this experiment, similar studies were conducted in which C3H mice were treated using a one-time vaccination, this time with PEPvIII-KLH in complete Freund's adjuvant as opposed to the DC vaccine. This vaccine protocol also resulted in increased median survival and ultimately long-term survival in nearly half of the mice (Heimberger et al.,

2003). Notably, mice with tumors that failed to exhibit responses to the PEPvIII-KLH vaccine were found to have IHC evidence of downregulated or completely absent EGFRvIII expression, suggesting that antigen escape variants may be associated with failure to adequately treat some tumors.

1.8.1.2 EGFRvIII: Clinical Studies

It has also been demonstrated in clinical trials that EGFRvIII-specific immunity can be induced with vaccines targeted against the EGFRvIII tumor specific antigen. A number of EGFRvIII-derived cytotoxic T lymphocyte (CTL) epitopes have been characterized to date (Wu et al., 2006), and previous data has shown that EGFRvIII-specific antibody titers, while absent in normal volunteers, may be detectable in patients with tumors expressing the mutant receptor (Purev et al., 2004). It is still unclear however whether cellular or humoral responses will ultimately provide the critical mediators for specific antitumor eradication using our approach.

The first clinical study evaluating the toxicity and potential efficacy of EGFRvIII-based vaccinations began with a Phase I trial (VICTORI) (Sampson et al., 2009) conducted at Duke University Medical Center (PI: John H. Sampson). Fifteen adults with newly diagnosed GBM (WHO grade III or IV) were enrolled in the study; criteria for eligibility did not include EGFRvIII expression. Of the fifteen patients, three did not ultimately receive vaccine due to progression of their tumors during external beam radiotherapy (ERBT). Following gross total tumor resection and completion of EBRT, 12

patients underwent leukapheresis to obtain peripheral blood mononuclear cells (PBMCs) in preparation for DC generation and immunologic monitoring. Prior to inoculation, DCs were pulsed for two hours with 500 μg PEPvIII peptide (Anaspec, San Jose CA) conjugated to KLH (Biosyn, Carlsbad CA). In total, patients received up to 1.1 x 10^8 DCs in three equal doses, injected intradermally every two weeks into the upper thigh, 10cm below the inguinal ligament. Patients were followed for toxicity and evidence of radiographic or clinical progression.

Patients in the VICTORI trial did not suffer serious adverse events exceeding Grade II toxicity at any DC dose tested (National Cancer Institute Common Toxicity Criteria). Blood drawn from patients following vaccination showed *ex vivo* evidence of antigen-specific cellular and humoral immune responses. Median survival for the 12 patients was 18.7 months after vaccination ($C.I._{95}$ 14.5, 25.6) and 22.8 months after histological diagnosis ($C.I._{95}$ 17.5, 29). These outcomes improve on what would have been expected by chance, according to Curran's recursive partition analysis (Curran et al., 1993). Eight of the 12 patients in this study belonged to group III, and the remaining 4 belonged to group IV, which have estimated survivals of 17.9 and 11.1 months, respectively. While 9 of the 12 patients in our study surpassed these estimates, the increase in survival was not statistically significant (P=0.083; binomial proportions), though these results may be negatively biased by the fact that EGFRvIII expression was not a criterion for inclusion in this Phase I toxicity trial.

Nevertheless, the outcomes associated with the DC-based, PEPvIII-specific vaccine were encouraging and warranted further testing at different centers; however the inherent cost and variability associated with autologous DC manufacturing made this approach impractical on a large scale. Thus, given the success of preclinical studies, a Phase II multicenter trial (ACTIVATE) (Heimberger et al., 2006) was attempted without the use of DCs, instead administering PEPvIII-KLH directly in combination with granulocyte macrophage-colony stimulating factor (GM-CSF).

ACTIVATE, a Phase II, multicenter clinical trial conducted at Duke (PI: John H. Sampson) and University of Texas, M.D. Anderson Cancer Center (PI: Amy B. Heimberger), enrolled 19 adults who all had EGFRvIII-expressing, newly-diagnosed primary GBM (WHO Grade IV). Prior to receiving the KLH-conjugated peptide vaccines, patients underwent >95% volumetric tumor resection, along with standard of care radiation therapy with concurrent TMZ. Vaccinations consisted of intradermal injections with 500μg PEPvIII-KLH (Anaspec, San Jose CA) and GM-CSF, administered near the inguinal region in the upper thigh, on alternating sides. The first three vaccines were given biweekly, followed by monthly injections until radiographic evidence of tumor progression or death.

Similar to what was observed in VICTORI, patients participating in ACTIVATE did not experience serious adverse events aside from local reactions at the injection site. This vaccine formulation was shown to elicit both humoral (Schmittling et al., 2008) and

delayed-type hypersensitivity immune responses specific for PEPvIII and EGFRvIII in a number of patients, and that detection of these responses predicts greater median overall survival (OS). Median time-to-progression (TTP) following surgery in patients who received the vaccine is 12 months (n=12), exceeding a median TTP of 7.1 months (n=29) calculated from a historical matched unvaccinated control group (p=0.0058). If and when tumors recurred, pathological samples were obtained and evaluated by IHC to determine EGFRvIII expression. Of the specimens examined in this trial, none were found to contain cells that display positive staining for EGFRvIII,

Following ACTIVATE, the ACT II trial was initiated (Sampson et al., 26: 2008 (May 20 suppl; abstr 2011)), which enrolled a total of 21 patients who essentially followed the same treatment scheme as those in ACTIVATE, except for the addition of two different TMZ dosing schedules concurrent with vaccination cycles; patients either received 200mg/m^2 TMZ x 5/28 days (ACT IIA [n=13]) or 100mg/m^2 TMZ x 21/28 days (ACT IIB [n=8]). While grade 2 TMZ-associated lymphopenia was observed in the majority of ACT II patients, it was found that all immune responses were unexpectedly either sustained or enhanced with successive TMZ treatments. The seemingly paradoxical relationship between TMZ-induced lymphopenia and improved PEPvIII-KLH-specific immunogenicity is currently under further investigation.

In summary, these trials to date collectively show that vaccination with a peptide containing the PEPvIII tumor epitope safely elicits a specific immune response against

EGFRvIII, and that this approach might be effective against cancers bearing the variant antigen. While the vaccine demonstrated significantly greater TTP and OS in GBM patients who have received the PEPvIII-KLH vaccine, definitive evidence for this promising effect will require confirmation from our ongoing randomized Phase III clinical trials.

1.9 Discussion

While the mechanisms underlying the beneficial effects of the EGFRvIII vaccine in patients with GBM are still unclear, it is our hope that additional experience with PEPvIII-KLH will elucidate our general understanding of various peptide vaccination strategies and their potential role in eliciting effective antitumor responses. A number of issues regarding the specific targeting of EGFRvIII by this peptide vaccine have yet to be addressed. It is known, for example, that although several tumors have been found to express the EGFRvIII mutation, cells within these cancers often exhibit significant antigenic heterogeneity. In addition, a major mechanism by which tumors have been shown to evade the immune system is through the process of immune editing, whereby tumor cells are selected for the ability to either mutate or down-regulate a targeted antigen of interest. Both of these phenomena likely contributed to observations in clinical studies of the EGFRvIII peptide vaccine, in which multiple patients achieved significant survival benefits but eventually recurred owing to the outgrowth of EGFRvIII antigen-loss variant tumors (Sampson et al., 2010). Unlike the EGFRvIII vaccine, which

was found to elicit a predominantly humoral immune response, strategies designed to promote EGFRvIII-targeted T-cell immunity in particular may actually address these therapeutic shortcomings. For instance, T-cell responses have been shown to mediate endogenous priming associated with epitope spreading (Pilon et al., 2003), and are also capable of protecting against antigen loss through direct bystander effects (Zhang et al., 2008). Given that recent clinical studies have associated favorable clinical responses with the ability to produce broad immunity to previously untargeted antigenic determinants (Corbiere et al., 2011), further study regarding the ability for T-cell based antitumor therapy to elicit these responses should be an emerging priority.

The following chapter will review in detail a particularly promising approach that seeks to harness the cytotoxic T-cell immune response against tumors through bispecific antibodies.

2 Bispecific Antibodies Engage T cells for Antitumor Immunotherapy

Although considerable evidence supports that T cells play a critical role in the immune response against cancer, the ability to mount and sustain tumor-specific cellular responses *in vivo* remains a challenge. A novel strategy that harnesses the cytotoxic advantage of T cell therapy is the use of bispecific antibodies designed to engage and activate endogenous polyclonal T cell populations *via* the CD3 complex, but only in the presence of a tumor antigen. Although antibody constructs with dual specificity were first described as anticancer therapeutics over 25 years ago, it was not until recently that one subclass of bispecific single-chain antibody, the bispecific T cell engager (BiTE), emerged as superior to previous iterations in achieving efficacy in both animal models and early clinical trials.

This chapter details the evolution of bispecific antibodies in antitumor immunotherapy, including insights as to their mechanism of action and data that support the BiTE platform as a therapeutic model with great potential in the treatment of malignant disease. Included will be a discussion regarding the greatest hurdles impeding further clinical translation of bispecific antibodies, specifically in the context of immunoprivileged sites as is the case for intracerebral malignancies.

2.1 Introduction to T-Cell Therapy for Cancer

Perhaps one of the clearest pieces of evidence in support of cellular-based cancer therapy is in the treatment of hematological malignancies such as leukemia and multiple myeloma, wherein individuals who relapsed after initial therapy with hemopoietic stem cell transplantation (HSCT) have ultimately achieved complete remission upon allogeneic infusion with unmanipulated donor lymphocytes (Dazzi et al., 2000, Lokhorst et al., 2000). In addition, following HSCT, positive clinical outcomes have been associated with the development of antigen-specific T cells reactive against either minor histocompatibility (Nishida et al., 2009) or tumor-associated antigens (Rezvani et al., 2007) in what has been described as a complex graft-versus-leukemia interaction (Sprangers et al., 2007, Kolb, 2008). While antitumor responses in this system are largely attributed to partial HLA-mismatch between donor and patient, these findings corroborate that under appropriate conditions, cellular immunity may be redirected therapeutically against neoplasms with remarkable efficacy.

In that vein, it has been established that—much like antibodies—T cells successfully penetrate deep tissue beds and target tumor antigen deposits therein with great specificity. Indeed, high densities of $CD8^+$ effector memory T cells have been isolated from tumor parenchyma in patients with colon cancer (Pages et al., 2005, Galon et al., 2006) and non-Hodgkin lymphoma (NHL) (Wahlin et al., 2007); moreover, the quantities of tumor infiltrating lymphocytes (TILs) were shown to predict increases in

overall survival, supporting the idea that these cell populations could be sequestered and then infused therapeutically. To that end, and as first described in a seminal paper by Rosenberg and colleagues in 1988 (Rosenberg et al., 1988), the adoptive transfer of autologous T cells derived from TIL isolates has demonstrated substantial clinical relevance, in one study leading to objective responses of up to 70% in patients with refractory metastatic melanoma (Dudley et al., 2005, Dudley et al., 2008).

With the hope of enhancing the efficacy of what already appears to be a potent T cell therapy, currently in development is the ability to engineer a patient's primary T cells with a second T cell receptor (TCR) of known specificity for a defined tumor antigen (Coccoris et al., 2005); early results from clinical studies of transgenic TCR technology confirm that this strategy may feasibly redirect and activate T cells against tumor antigens (Duval et al., 2006). Based on similar principles, T cells have also been modified to express synthetic chimeric antigen receptors (CARs) which consist of an extracellular single chain variable fragment (scFv) derived from a tumor-reactive monoclonal antibody, fused to an intracellular CD3 ζ chain signaling domain (Cartellieri et al., 2010), thereby endowing T cells with expansive reactive capabilities that span what would otherwise be physiologically exclusive to the humoral repertoire. Although it remains unclear whether T cell modifications such as transgenic TCRs and CARs will ultimately realize widespread clinical success, these advancements in adoptive therapy

continue to show promise in early clinical trials, supporting the general notion that T cells are indeed critical mediators in an effective antitumor immune response.

2.1.1 Updates on Chimeric Antigen Receptor Therapy

Chimeric antigen receptors (CARs) represent a versatile class of receptors that are generated by combining the variable region of an antibody with a T-cell signaling molecule, usually CD3 (Gross et al., 1989). Because their capacity for antigen recognition is derived from antibody binding, CARs have the ability to mimic endogenous TCR-mediated activation, without the need for classical MHC restriction. Moreover, whereas physiological TCRs are restricted by thymic selection, antibody-redirected CARs can accommodate virtually infinite antigenic diversity and operate at affinities even in the nanomolar range (Hollyman et al., 2009, Lamers et al., 2006).

An additional advantage of the CAR platform is the incorporation of costimulatory molecules such as CD28 and 4-1BB into the CD3 signaling domain to improve T-cell expansion, survival, cytokine secretion and tumor lysis (Milone et al., 2009, Zhao et al., 2009, Zhong et al., 2010). Clinical trials utilizing these second and third generation CARs have now targeted a variety of antigens and malignancies and have demonstrated their remarkable potential (Pule et al., 2008, Kochenderfer et al., 2012, Porter et al., 2011, Brentjens et al., 2011). However, severe adverse events and even patient deaths have occurred when these CARs have been directed against antigens

shared by normal tissues including CD19 and ERBB2 (Morgan et al., 2010, Brentjens et al., 2010).

2.2 Current Challenges for T-Cell Antitumor Responses

Despite their success to date, T cell-based therapies as they presently exist are limited by a number of conceivable drawbacks. First, adoptive cell transfer (ACT) is by nature highly personalized and thus labor-intensive, requiring that laboratories not only possess an extraordinary level of expertise, but also that they create essentially new reagents for each patient who is to receive treatment. Furthermore, besides melanoma, few cancers have been shown to naturally give rise to tumor-reactive lymphocytes that can be isolated and productively expanded in every eligible patient, bringing into question the practicality of applying ACT across variegated clinical scenarios.

Perhaps the most discouraging aspect of ACT, however, lies in its fundamental dependence on the *in vivo* maintenance of infused T cell populations. The immunological processes that regulate survival and proliferation of engrafted lymphocytes are complex and tightly controlled, though this balance is often highly dysregulated in a typical cancer patient. Late-stage tumors in particular exhibit a number of evasive mechanisms that antagonize the development of an optimal cellular response; during a process known as immunoediting, cancer cells are hypothesized to undergo Darwinian-like selection for characteristics that obstruct T cell reactivity, such as downregulation of co-stimulatory molecules, MHC, and other factors closely

associated with antigen presentation (Marincola et al., 2000, Meidenbauer et al., 2004). Tumors are also known to alter earlier events in antigen processing, as evidenced by their derangement of normal proteasomal machinery (Seliger et al., 2000, Gobbi et al., 2004, Hayashi and Faustman, 2002), loss of intracellular peptide transporters (Johnsen et al., 1999, Ritz et al., 2001), or inactivation of β_2-microglobulin protein (Cabrera et al., 2003), a necessary component for transport of MHC class I to the cell surface.

Regardless of the precise mechanism, one consequence of immune escape is that tumor-specific lymphocytes, even those with highly avid, genetically modified TCRs, will not receive adequate stimuli to be activated or sustained *in vivo*. It has been demonstrated, for instance, that without appropriate co-stimulation signals, first-generation CAR-transduced T cells fail to proliferate and survive following infusion, leading to only modest results in early clinical trials (Kershaw et al., 2006, Lamers et al., 2006, Till et al., 2008). To address this issue, the latest CARs have been modified to incorporate additional pro-immunomodulatory signaling endodomains from costimulatory molecules like CD28 and CD137 (Cartellieri et al., 2010, Zhao et al., 2009). While such second- and third-generation constructs have yet to be validated in clinical trials, it has already been forewarned that expression of manifold signaling moieties within a single lymphocyte clone may excessively reduce the threshold of activation, leading to increased risk for autoimmune toxicity.

Indeed, potentially devastating effects associated with non-specific lymphocyte activation have been clearly illustrated in the early discontinuation of a clinical trial studying TGN1412, a humanized anti-CD28 superantagonistic monoclonal antibody uniquely designed to activate T cells without the need for concomitant TCR engagement (Suntharalingam et al., 2006). Immediately after intravenous infusion with TGN1412, all six study participants experienced severe cytokine storm and were admitted to intensive care for multisystem organ failure. While multiple factors may have ultimately contributed to these disastrous outcomes, the magnitude of systemic toxicities observed in this setting reaffirms the critical importance of maintaining targeted specificity.

The overall challenge, then, is to develop safer, more specific and more effective antitumor therapies that not only exploit the cytotoxic potential of the cellular immune response and circumvent mechanisms of tumor evasion, but are also manufactured with relative ease and possess broad applicability across clinical scenarios. To these ends, the bispecific antibody therapeutic platform represents a promising approach, as it has been shown to successfully engage and activate endogenous polyclonal T cells *via* the TCR-CD3 complex, exclusively in the presence of a tumor antigen.

2.3 The History and Development of Bispecific Antibodies

Bispecific antibodies were first developed upon previously established principles of monoclonal antibody therapy—namely that, in the treatment of malignant diseases, antibodies had already been shown to possess the specificity necessary to mediate a

number of antigen-specific immune mechanisms. Native antibodies of the immunoglobulin G (IgG) type for example are made up of two identical, antigen-binding, variable regions joined by a constant fragment domain (Fc). Monocytes and other phagocytic cells bind Fc domains *via* their surface Fcγ receptors, resulting in specific lysis of targeted cells through a well-characterized antibody-dependent cell-mediated cytotoxicity (ADCC) mechanism. Besides interacting with cellular effectors however, antibodies also recruit endogenous proteins of the complement cascade, or alternatively, they can be artificially modified to deliver a payload of cytotoxic molecules including radioisotopes (Wiseman et al., 2002, Zelenetz, 2003), chemotherapies (Sievers et al., 2001), or bacterial toxins (Oh et al., 2009) to tumors with great specificity.

Like armed monoclonal antibodies, bispecific antibodies do not occur naturally in the human body and must be created using either recombinant DNA or cell-fusion technologies. These techniques make it possible to combine multiple humoral specificities into a single molecule while retaining the specificity—and occasionally the function—of each component contributing to its overall design. Thus, in the most general sense, the term "bispecific antibody" refers to a class of constructs in which two antibody-derived antigen-specific binding sites are aligned within one molecule. In the context of tumor immunotherapy, this typically means that one arm of the construct is specific for an epitope on the surface of a cancer cell (target-binding arm), while the

other arm is specific for the effector cell (effector-binding arm). The theoretical benefit of this divalent design lies in its potential to simultaneously redirect and locally activate cellular effectors in the presence of cancer cells, thereby maximizing proximate target lysis while minimizing non-specific cytotoxicity in surrounding healthy cells and tissues.

The concept of using bispecific antibodies to activate T cells against tumor antigen was first described over 20 years ago (Perez et al., 1985, Staerz et al., 1985). Among the first formats pursued were constructs designed to bind the monomorphic TCR-CD3 complex, a strategy that offers a number of conceivable advantages. Triggering lysis by this approach allows bispecific antibodies to interact globally with the T cell compartment, thereby circumventing the restriction of clonotypic specificity and proliferation. In addition, because the target-binding arm is derived at least in part from the variable portion of a tumor-specific antibody, the overall effect is not only highly specific, but also widely applicable against a broad array of antigens—that is, antibodies possess the ability to bind tumor epitopes beyond the MHC-peptide complexes classically recognized by TCR. As a result, bispecific antibodies both increase the complement of targetable T cell antigens and also overcome mechanisms of tumor immune escape such as loss or downregulation of MHC.

To date, technical aspects of producing bispecific antibody constructs at a clinically relevant quantity and purity have proven challenging. As a result, a wide variety of formats based on the same functional principle have been pursued; these

include diabodies, tandem scFv, cross-linked antigen binding fragments (Fab), and quadroma-derived constructs.

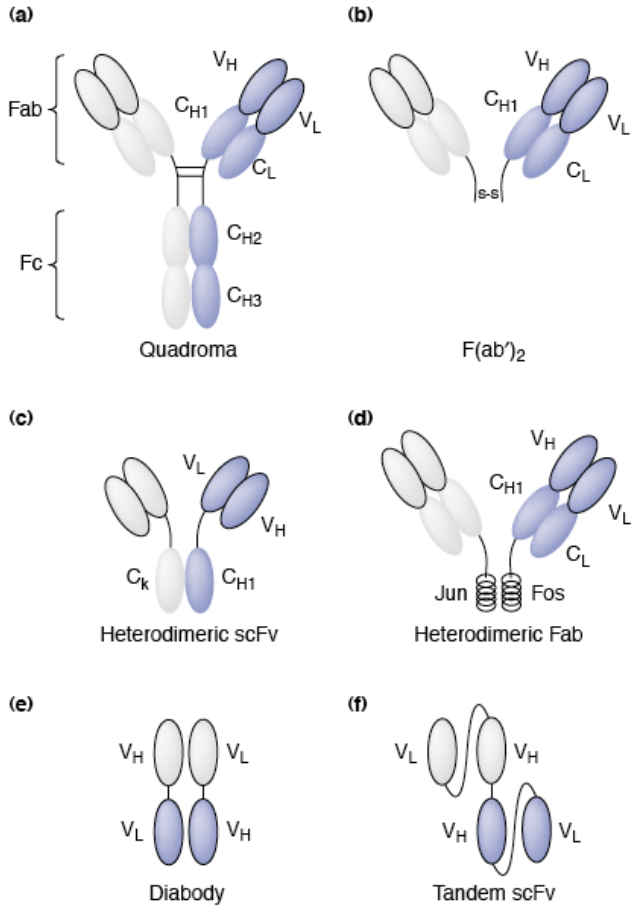


Figure 4: Bispecific antibody formats.
(a) Quadroma-derived bispecific antibody, resembling monospecific monoclonal antibodies in structure and size. (b–d) Heterodimeric bispecific antibodies of medium size, comprising constant immunoglobulin regions and/or heterodimerization domains. (e–f) Bispecific antibodies of minimal size, consisting only of variable immunoglobulin regions. Abbreviations: C_{H1-3}, constant regions of Ig-heavy chain; C_L, constant regions of Ig-light chain; Fab, antigen binding fragment; Fc, constant fragment; V_H, variable region of Ig-heavy chain; V_L, variable region of Ig-light chain. The difference in colors (i.e. gray and purple) represents different binding specificities. Figure reproduced with permission (Kufer et al., 2004).

The first attempt to produce antibodies with dual specificity employed quadroma technology, which relies on the fusion of two distinct hybridomas expressing monoclonal antibodies of desired specificity (Staerz and Bevan, 1986, Milstein and Cuello, 1983, Suresh et al., 1986). Within these hybrid-hybridomas, bispecific antibodies are produced through the random pairing of immunoglobulin (Ig) heavy and light chains. Because unfunctional, mispaired byproducts often result during this process and subsequently confound purification, more recent methods have been developed to employ antibody chains selected for their tendency to couple with each other preferentially (Lindhofer et al., 1995). Structurally, quadroma-derived bispecific antibodies resemble conventional antibodies; however, in order to avoid the potentially undesirable or unpredictable effects of an intact constant region, the Fc domain may be cleaved away enzymatically, leaving behind paired Fab fragments with distinct antigen specificity (Mezzanzanica et al., 1988).

Similar divalent constructs have been produced without the use of quadroma technology, either by site-directed chemical cross-linking between two Fab fragments at hinge cysteine residues (Glennie et al., 1987), or by various recombinant DNA strategies that introduce moieties designed to promote favorable pairing *via* specific inter-protein interactions. For example, in order to optimize heterodimer formation and reduce mispairing events, constant fragments of individual chains can be mutated to express adhesive amino acid sequences (Merchant et al., 1998), and in some cases modified or

completely replaced with complementary proteins pairs (*e.g.* Fos and Jun, or Ig domains C_κ and C_{H1}) that exhibit natural tendencies to associate as heterodimers (Kostelny et al., 1992, Muller et al., 1998, de Kruif and Logtenberg, 1996).

Among the smallest bispecific antibody constructs are diabodies and tandem scFvs, both of which represent unique combinations and orientations of antibody variable domains (V_H and V_L) within a single molecule. The basic diabody structure consists of two separate single chain fusion products held together by non-covalent interactions. Each single chain contains one V_H and one V_L domain with opposing specificities (Holliger et al., 1993, Holliger et al., 1996). When co-expressed in bacteria, independent single chains combine with each other to form stable hybrid bispecific diabody heterodimers. However, as a pairing byproduct, it is again possible to produce an inactive homodimer; thus, in order to improve overall stability as well as purity, tandem diabodies were designed, in which all four variable domains are translated as a single chain molecule (Kipriyanov et al., 1999). There still exists a theoretical concern, however, that inherent to the diabody approach is a structural and steric limitation related to the number of amino acid linkers connecting antigen-binding arms. Whereas diabodies contain two separate linkers between each arm, bispecific tandem scFv molecules, by contrast, include only one flexible glycine-serine linker between native scFv domains. In other words, tandem scFvs possess two binding sites that can rotate freely relative to each other, offering conceivable advantages in settings where antigen

epitopes of interest have limited accessibility on different cell surfaces. Like diabodies, production of tandem scFvs was also first attempted in *Escherichia coli* systems (Mallender and Voss, 1994, Gruber et al., 1994), though studies would reveal that bacterial expression for bispecific tandem scFv was unreliable (Kipriyanov et al., 2003), and that yields of functional product remained poor following renaturation of proteins isolated from inclusion bodies. This issue, however, was largely addressed after establishing that mammalian cells (Schlatter et al., 2005) could be used to manufacture bispecific tandem scFvs with relative ease (Birch and Racher, 2006), since the proteins are secreted directly into culture media in functional, folded conformations (Mack et al., 1995).

To date, few studies have presented thorough comparative analyses between tandem diabody and scFv formats. Early evidence suggests that tandem diabodies may exhibit increased stability due to their tightly packed variable domains, though as previously mentioned, this conformation conversely restricts mobility at the linker region. In a recent report, both types of tandem bispecific antibody demonstrated indistinguishable binding kinetics and biological activity *in vitro* when targeting carcinoembryonic antigen-expressing tumor cells (Korn et al., 2004). Other groups have shown that conversion from preexisting diabody constructs to tandem scFv may drastically increase cytotoxic function (Asano et al., 2011). Additional studies are necessary to corroborate these observations *in vivo* and multiple factors will ultimately

dictate whether either format will translate more successfully into the clinic. Perhaps the relative dearth of literature contrasting the therapeutic potential of what may at first appear to be largely synonymous antibody-derived conjugates and fragments reflects the rapidity with which several antibody-modifying technologies have become available and subsequently cultivated by the pharmaceutical industry. As such, more than ten of the largest pharma companies have either developed or licensed a unique variant of the bispecific antibody theme, and a timely review of selected initiatives has been published elsewhere (Moran, 2011). Notably, early preclinical data suggests that one platform in particular, the dual-affinity re-targeting (DART) molecule—distinguished by the addition of a proprietary covalent linkage between V_H domains—may improve upon the stability and potency realized by previous diabody and scFv formats (Moore et al., 2011).

2.4 Clinical Experience with Bispecific Antibodies

While a vast majority of bispecific antibodies have been developed to activate T cells against tumor-associated antigens, efforts have also been made to redirect other cellular effectors of the immune system for targeted cytolysis. Among myeloid cells, monocytes and neutrophils have been shown to mediate antitumor effects when triggered by chemically linked $F(ab')_2$ bispecific antibodies through either CD64 (James et al., 2001) or CD89 (Deo et al., 1998) activation signaling, respectively. Limited clinical efficacy has been reported to date on these constructs, though this might be explained by

the fact that corresponding preclinical studies required extremely high effector-to-target (E:T) ratios (up to 200:1), high concentrations of the therapeutic agent (from 0.1 to 1 μg ml^{-1}), and in one case prestimulation of effector cells with IFNγ, in order to achieve acceptable efficacy.

A number of notable quadroma-derived bispecific antibody constructs have been created and tested for their ability to engage natural killer (NK) cells *via* CD16 in the presence of Her2/neu- and CD30-expressing tumors (Weiner et al., 1995, Hartmann et al., 1997). In what is perhaps the first study establishing clinical proof for the bispecific antibody concept, treatment with a CD16-activating $F(ab')_2$ that targets CD30 in patients with Hodgkin's disease was shown to lead to objective antitumor responses in 25% of patients, including one documented complete remission and three partial remissions (Hartmann et al., 2001). While initial study results showed promise, it is important to note that these trials ultimately had to be halted due to inadequate production of clinical test material.

As previously mentioned, in order to redirect and activate T cells—particularly CTLs—against tumors, the most popular approach by far is triggering *via* the TCR-associated CD3 complex. Under normal physiological conditions, stimulation of T cell activity occurs through a series of fundamental processes regulated by several molecules; traditionally, a first signal is provided by TCR engagement with peptide-MHC, and a second signal arises from interactions with costimulatory molecules on the

surface of the T cell, including CD28. Therefore, it was not surprising to discover that, at first, most CD3-directed bispecific $F(ab')_2$ antibodies and recombinant diabodies demonstrated a strict requirement for lymphocyte preconditioning or costimulation in order to elicit significant lysis against tumor cells both *in vitro* and in animal models (Demanet et al., 1996, Titus et al., 1987, Weiner and Hillstrom, 1991, Renner et al., 1994, Kipriyanov et al., 1998, Zhu et al., 1996, Cochlovius et al., 2000). In addition, until the advent of tandem scFv formats, it was unclear whether systemic therapy with CD3-engaging bispecific antibodies could feasibly treat metastatic disease, given that previous efforts to administer constructs intravenously required excessive dosing and resulted in prohibitive autoimmune toxicity (Kroesen et al., 1994, De Gast et al., 1995).

Consequently, while bispecific antibodies in general tout an extensive history, even the most potently activating molecules developed to date have been thwarted by several shortcomings, among which include an inability to produce the drug sufficient quantities, an apparent need for T cell costimulation, and a risk of limiting side-effects at high doses.

Promising to deliver where previous iterations have fallen short, however, is a new subclass of recombinant bispecific tandem scFv referred to as the bispecific T cell engager (BiTE). Because of its recent success in animal models and early clinical trials, the BiTE format has rekindled great interest in the overall development of antibody-

directed, antitumor T cell therapies, and may offer wide applications across multiple human cancers in upcoming years.

2.5 Bispecific T-Cell Engagers Redirect Cellular Immunity Against Cancer

Among the hundreds of relatively unsuccessful bispecific antibody designs, BiTEs have emerged as superior to other formats; they are relatively stable and easy to produce in mammalian cells, are extremely potent at low doses, and are able to mediate cytotoxicity in the absence of supplemental lymphocyte stimulation (Loffler et al., 2000, Kufer et al., 1997, Mack et al., 1997, Mack et al., 1995). Generally, BiTEs are polypeptides consisting of two scFvs in tandem with total molecular weights ranging from 55 to 60 kDa; by definition, while their effector-binding arms bind specifically to the epsilon subunit of CD3, their opposing target-binding arms can be directed against any number of epitopes, such as those differentially expressed on the surface of a tumor cell.

In lymphoma, one prominent, well-characterized BiTE is the recombinant bscCD19xCD3 construct, targeting the pan B-cell antigen, CD19 (Bargou et al., 2008). Human xenograft murine models, in which severe combined immunodeficiency, non-obese diabetic (NOD/SCID) mice are engrafted with human T-cells and tumor cells, have been used extensively to study the activity of the bscCD19xCD3 BiTE *in vivo* with remarkable results; treatment prevented growth of subcutaneous human B lymphoma xenografts and in some cases even cured animals with early stage tumors (Dreier et al.,

2003). Using the same murine model, another group of successful studies examined the efficacy of BiTEs directed against EpCAM (Schlereth et al., 2006, Brischwein et al., 2006), an epithelial cell adhesion molecule overexpressed in both colon and ovarian cancers. Additional tumor antigens that are currently being investigated as targets for BiTE therapy include Her2/neu, EGFR, CEA, CD33, EphA2, and MCSP (Baeuerle and Reinhardt, 2009).

Most recently, translation to human trials assessing the clinical activity of the bscCD19xCD3 construct in patients with non-Hodgkin's lymphoma have confirmed its remarkable potency (Bargou et al., 2008, Handgretinger et al., 2011). All seven patients in the study who received continuous infusion with BiTE at 0.06 mg/m^2 per day showed objective tumor regression, including five partial responses and two complete responses with clearance of tumor from the blood, bone marrow, and liver. This drug dose produced serum levels five orders of magnitude lower than reported effective doses of the CD19-specific standard-of-care, rituximab.

Notably, no dose-limiting cytokine release syndrome was evident in this trial. Although CNS side effects did occur (*e.g.*, confusion, tremor, focal neurologic deficits and seizures) they were all fully reversible. Similarly, autoimmune phenomena were generally absent; however treatment with the bscCD19xCD3 BiTE led to nearly complete depletion of CD19-expressing normal B-cells.

The second BiTE construct that has been tested in clinical trials is MT110, designed to target EpCAM on a wide array of solid tumors in patients with lung, gastric, and colorectal cancers. Early phase I study results demonstrated disease stabilization in 7 of 19 evaluable patients following infusion with clinically-tolerated low doses of MT110, and evaluation of the therapy at higher doses is ongoing (Fiedler, 2009).

2.6 Mechanistic Studies of BiTE-Mediated Antitumor Immunity

Part of the reason why it is thought that BiTEs so potently mediate T cell activation is that, on a cellular level, they have been shown to induce immunological synapses between $CD8^+$ T cells and tumor cells that are indistinguishable in composition, size, and subdomain arrangement from native cytolytic synapses (Offner et al., 2006). Following antigen-specific BiTE-mediated activation, apoptosis occurs *via* perforin-mediated membrane disruption, followed by influx of various granzyme proteases (Haas et al., 2009). Also, T cells begin to proliferate, secrete characteristic cytokines, including TNFα, IFNγ, IL-6, IL-2, IL-4 and IL-10, and express surface markers that indicate activation such as CD69 and CD25. While proliferation in response to BiTE activation is most obvious in the $CD8^+$ T cell compartment, $CD4^+$ cells are also known to respond by dramatically upregulating expression of granzyme B (Mack et al., 1997), a critical protein involved in the apoptotic process.

BiTE proteins have the ability to initiate lytic synapses in the presence of tumor antigen, and experiments have demonstrated that robust T cell responses are possible

even against tumor cells that do not express MHC class I. Interestingly, BiTEs have also been shown to reactivate potentially unresponsive T cells, effectively redirecting TIL populations to eradicate donor-matched xenotransplanted tumors in an immunocompromised mouse model (Miescher et al., 1986, Schlereth et al., 2005).

One study using video-assisted microscopy showed that during the cytotoxic event, BiTE constructs transiently link T cells with tumor cells and sustain repeated rounds of serial target cell lysis at E:T ratios as low as 1:5 (Hoffmann et al., 2005). These findings are consistent with the fact that, unlike previous bispecific antibody formats, BiTEs can trigger specific lysis at concentrations even in the low picomolar range (Dreier et al., 2002). At the same time, BiTEs nevertheless display exquisite specificity; at concentrations several thousand-fold greater than their EC_{50} (2-1,000 pg/mL) (Baeuerle et al., 2009), BiTEs retain their specificity and will not trigger T cell activation unless presented with a substrate expressing the target antigen (Brischwein et al., 2007).

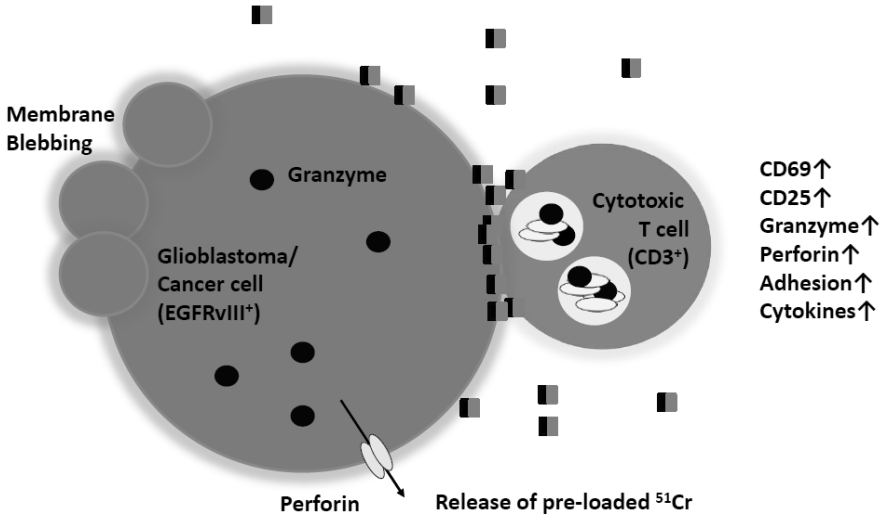


Figure 5: Schematic summary of BiTE mechanism-of-action.

2.7 Discussion for Chapters 1 and 2

More than 100 years ago, Paul Ehrlich first suggested that the immune system could be redirected to recognize and eradicate tumors (Ehrlich, 1906), and since that time, parallel advances in both technology and our basic understanding of immunological principles have made it possible to manipulate the human immune system against cancer with unprecedented elegance.

When compared with alternative T cell-based therapies for cancer, BiTEs represent a unique approach in that they are fully antibody-derived and, as such, do not require the *ex vivo* enrichment and modification, nor the *in vivo* maintenance associated with antitumor therapies that rely on infused lymphocytes. Moreover, BiTEs maintain the ability to mediate tumor lysis despite certain classical mechanisms of immune

escape, given that they interact with polyclonal T cells without requirements for MHC presentation or TCR specificity. It has also been suggested that monovalent interactions between surface antigen and bispecific constructs are less likely to induce antigenic modulation than binding by parental monoclonal antibodies (Nolan and O'Kennedy, 1990).

In contrast to preceding bispecific antibody formats, BiTEs clearly possess multiple advantages that span across nearly all characteristics from structure to function. As previously mentioned, in the case of bscCD19xCD3, targeted lysis was potent enough such that global eradication of even normal CD19-expressing cells occurred. Thus, perhaps one limitation to realizing the maximum therapeutic potential of the BiTE platform is a lack of tumor-specific targets that are both frequently and homogenously expressed in tumor tissue.

Among the many mutations that often appear in tumor cells, the type I epidermal growth factor receptor (EGFR) is perhaps the most widely implicated. As reviewed in Chapter 1 of this dissertation, one well-characterized mutant form of the receptor is the class III variant, EGFRvIII, a constitutively active, ligand-independent version of the wild-type receptor, first discovered in human glioblastoma (GBM) but subsequently identified in a wide variety of cancers. Our group has extensive experience with EGFRvIII, which—because of its exclusive expression in tumor cells and complete absence from normal, healthy tissues—offers an ideal extracellular epitope for

antibody-based therapies. Until our studies, there have not been published studies of a BiTE designed to target the EGFRvIII antigen or any other tumor-specific antigen.

Because our laboratory focuses primarly on intracerebral malignancies and immunotherapy for glioma, we are intimately familiar with the challenges associated with administering therapeutic effector cells and molecules in the context of an intact blood brain barrier (BBB) and intratumoral pressures that might hinder drug diffusion or delivery. A number of factors must be considered when addressing whether systemically administered treatments will reach a tumor within the brain. While it is believed that, under normal physiological conditions, the BBB is poorly permeable to both antibodies and naïve T lymphocytes, it has long been established that in the presence of neuroinflammation, the BBB has diminished ability to block the migration of cells and proteins into the CNS (de Vries et al., 1997). How this phenomenon will impact the ability for BiTEs to localize to an intracerebral tumor is currently unknown. Additionally, whether BiTEs will reactivate TILs within glioma tissue, or possibly facilitate trafficking of T cells from the periphery across the BBB, has yet to be studied. The potential inhibitory impact of intratumoral regulatory T cells will also need to be considered. These questions in will be addressed by several experiments throughout the remainder of this dissertation.

Despite burgeoning interest in bispecific antibodies and permutations thereof, the issues of stability and cost-effective production persist as major obstacles for those

who wish to ultimately advance this therapeutic platform. Given that current clinical protocols for bispecific therapeutics inconveniently require continuous infusion over several weeks, as the field continues to mature, priorities will converge on molecular modifications that are designed to enhance half-life and maintenance at clinically relevant plasma concentrations. Furthermore, the latest technologies designed to optimize production and reduce manufacturing costs of monoclonal antibodies and their derivatives in insect (Johansson et al., 2007) and mammalian cells may help to propel development of bispecific antibodies in upcoming years.

3 Generation of Tandem Single-Chain Variable Fragment Reagents for Bispecific Antibody Evaluation

3.1 Introduction

As reviewed in the preceding chapter, the term bispecific antibody can refer to any one of many antibody-based molecules that have the capacity to recognize two different antigens. The BiTE platform is perhaps one of the most successful subclasses of bispecific antibody given that it has proven feasible to manufacture and administer on a large scale, sufficient at least for early translation in clinical trials. Despite this success, the ability to efficiently produce bispecific antibody reagents in general remains a paramount challenge in the field (Kufer et al., 2004).

Among the most popular formats for bispecific antibodies are diabodies and tandem scFv, the latter of which is essentially synonymous with BiTE. Both the diabody format and the tandem scFv molecule have recently become popularized owing to their small size and relative ease of manipulation using standard recombinant technologies. In the case of diabodies, another advantage is their ability to be produced at large quantities in bacterial expression systems. To date, there has been limited experience producing tandem scFv molecules in bacterial expression systems like *Escherichia coli*, and it appears that, unlike diabodies, several groups have reported difficulty refolding tandem scFv molecules from insoluble inclusion bodies, specifically due to the unpredictable fashion by which variable domains fold and associate with each other in

solution (Kipriyanov et al., 2003, Mallender and Voss, 1994, Gruber et al., 1994). Notably, because tandem scFvs typically possess four variable domains, these molecules have the added option of folding either between adjacent domains or head-to-toe, much like what has been described elsewhere for the bispecific single chain diabody.

In the following sections we will introduce our design for the EGFRvIII-specific BiTE. In addition, despite the known challenges of producing functional tandem scFv molecules in bacterial systems, we will demonstrate the efficient expression and purification of several bispecific antibodies of the BiTE subclass, which will serve as the basis for reagents utilized throughout the remainder of this dissertation.

3.2 Materials and Methods

3.2.1 Construction and Expression of cDNA Encoding bscEGFRvIIIxCD3

Synthesis of cDNA encoding bscEGFRvIIIxCD3 was carried out using standard recombinant technologies. The scFv directed against EGFRvIII was isolated from pMR1-1(scFv) (Kuan et al., 1999), and the scFv specific for human CD3 was derived from the hybridoma cell line, OKT3, by previously described methods (Arakawa et al., 1996). A hexahistidine-tag was added to the carboxyl terminus to aid in the detection and purification of the final protein product. To obtain the bscEGFRvIIIxCD3-encoding DNA, overlapping PCR was performed with primers MR1-1 FW, MR1-1 RV, OKT3 scFv FW and OKT3scFv RV. With a linker sequence at the 3′ of MR1-1 and the 5′ of OKT3

scFv, the two scFvs can be annealed together using the MR1-1 FW and OKT3 scFv RV primers.

Table 3: Primers designed to introduce a flexible linker and construct bscEGFRvIIIxCD3 by overlapping PCR.

Designation	Sequence
MR1-1 FW	CGATCATATGGCCATGGCCCAG GTGAAACTGCAGCAGTCT
MR1-1 RV	AGACTGCTGCAGCTGGACCTGTGAACCGCCACC ACCTTTGATTTCCAGCTTGGTGCCATC
OKT3 scFv FW	GATGGCACCAAGCTGGAAATCAAAGGTGGTGGCGGTTCAC AGGTCCAGCTGCAGCAGTCT
OKT3 scFv RV	CAGTGAATTCTTAGTGGTGATGGTGATGATGGTTTATTTCC AACTTTGTCCCCGA

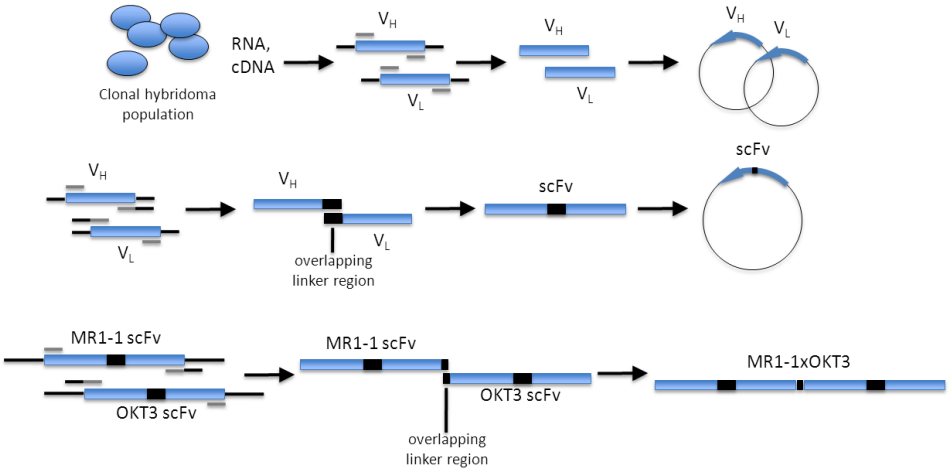


Figure 6: Schema of MR1-1xOKT3 (cDNA for bscEGFRvIIIxCD3) design.

The PCR product was analyzed on a standard agarose-gel system and purified according to manufacturer's instructions (QIAquick Gel Extraction Kit, QIAGEN). The resulting gene was cloned into pGEM-T Easy vector (Promega) sequenced and

subcloned into the expression vector, pRB199 (Kuan et al., 2000) *via* NdeI and EcoRI restriction sites.

```
caggtgaaactgcagcagtctgggggaggcttagtgaagcctggagcgtctctgaaactc
 Q  V  K  L  Q  Q  S  G  G  G  L  V  K  P  G  A  S  L  K  L
tcctgtgtaacctctggattcactttcagaaaatttggcatgtcttgggttcgccagact
 S  C  V  T  S  G  F  T  F  R  K  F  G  M  S  W  V  R  Q  T
tcagacaagaggctggaatgggtcgcatccattagtactggcggttataatacctactat
 S  D  K  R  L  E  W  V  A  S  I  S  T  G  G  Y  N  T  Y  Y
tcagacaatgtaaagggccgattcaccatctccagagagaatgccaagaacaccctgtac
 S  D  N  V  K  G  R  F  T  I  S  R  E  N  A  K  N  T  L  Y
ctgcaaatgagtagtctgaagtctgaggacacggccttgtattactgcacaagaggctat
 L  Q  M  S  S  L  K  S  E  D  T  A  L  Y  Y  C  T  R  G  Y
tctccgtacagctatgctatggactactggggccaagggaccacggtcaccgtctcctca
 S  P  Y  S  Y  A  M  D  Y  W  G  Q  G  T  T  V  T  V  S  S
agtggaggcggttcaggcggaggtggctctggcggtggcggatcggacatcgagctcact
 S  G  G  G  S  G  G  G  G  S  G  G  G  G  S  D  I  E  L  T
cagtctccagcatccctgtccgtggctacaggagaaaaagccactatcagatgcatgacc
 Q  S  P  A  S  L  S  V  A  T  G  E  K  A  T  I  R  C  M  T
agcactgatattgatgatgatatgaactggtaccagcagaagccaggggaacctcctaag
 S  T  D  I  D  D  D  M  N  W  Y  Q  Q  K  P  G  E  P  P  K
ttccttatttcagaaggcaatactcttcgtcctggagtcccatcccgattttccagcagt
 F  L  I  S  E  G  N  T  L  R  P  G  V  P  S  R  F  S  S  S
ggcactggcacagattttgtttttacaattgaaaacacactctcggaagatgttggtgat
 G  T  G  T  D  F  V  F  T  I  E  N  T  L  S  E  D  V  G  D
tactactgtttgcaatcctggaacgtgcctcttacattcggtgatggcaccaagctggaa
 Y  Y  C  L  Q  S  W  N  V  P  L  T  F  G  D  G  T  K  L  E
atcaaaggtggtggcggttcacaggtccagctgcagcagtctggggctgaactggcaaga
 I  K  G  G  G  G  S  Q  V  Q  L  Q  Q  S  G  A  E  L  A  R
cctggggcctcagtgaagatgtcctgcaaggcttctggctacacctttactaggtacacg
 P  G  A  S  V  K  M  S  C  K  A  S  G  Y  T  F  T  R  Y  T
atgcactgggtaaaacagaggcctggacagggtctggaatggattggatacattaatcct
 M  H  W  V  K  Q  R  P  G  Q  G  L  E  W  I  G  Y  I  N  P
agccgtggttatactaattacaatcagaagttcaaggacaaggccacattgactacagac
 S  R  G  Y  T  N  Y  N  Q  K  F  K  D  K  A  T  L  T  T  D
aaatcctccagcacagcctacatgcaactgagcagcctgacatctgaggactctgcagtc
 K  S  S  S  T  A  Y  M  Q  L  S  S  L  T  S  E  D  S  A  V
tattactgtgcaagatattatgatgatcattactgccttgactactggggccaaggcacc
 Y  Y  C  A  R  Y  Y  D  D  H  Y  C  L  D  Y  W  G  Q  G  T
actctcacagtctcctcaggtggtggcggttcaggcggaggtggctctggcggtggcgga
 T  L  T  V  S  S  G  G  G  G  S  G  G  G  G  S  G  G  G  G
tcgcaaattgttctcacccagtctccagcaatcatgtctgcatctccaggggagaaggtc
 S  Q  I  V  L  T  Q  S  P  A  I  M  S  A  S  P  G  E  K  V
accatgacctgcagtgccagctcaagtgtaagttacatgaactggtaccagcagaagtca
 T  M  T  C  S  A  S  S  S  V  S  Y  M  N  W  Y  Q  Q  K  S
ggcacctcccccaaaagatggatttatgacacatccaaactggcttctggagtccctgct
 G  T  S  P  K  R  W  I  Y  D  T  S  K  L  A  S  G  V  P  A
cacttcaggggcagtgggtctgggacctcttactctctcacaatcagcggcatggaggct
 H  F  R  G  S  G  S  G  T  S  Y  S  L  T  I  S  G  M  E  A
gaagatgctgccacttattactgccagcagtggagtagtaacccattcacgttcggctcg
 E  D  A  A  T  Y  Y  C  Q  Q  W  S  S  N  P  F  T  F  G  S
gggacaaagttggaaataaac
 G  T  K  L  E  I  N
```

Figure 7: cDNA and deduced amino acid sequence of the murine EGFRvIII BiTE

3.2.2 Expression and Isolation of Insoluble Inclusion Bodies

Bispecific antibody-encoding cDNA in pRB199 was transformed into *E. coli* strain BL21(λDE3). Bacterial cultures were inoculated into Terrific Broth Complete (Teknova) containing 80 μg/mL chloramphenicol and grown at 37 °C to an A_{600} of 2.0. To prepare culture media, 1 mL of a stock chloramphenicol solution was added to each liter of Terrific Broth Complete, along with 20 mL of 20% glucose solution and 4.1 mL of a 1 M MgSO4 solution. Prepared media was divided equally into six 2 L flasks (500 mL per 2 L flask). Expression of the recombinant bispecific antibody was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C for 4 hours. Cultures were centrifuged and bacterial pellets were resuspended in a buffer containing 50 mM Tris, pH 7.5, 20 mM EDTA (TE50/20) for storage at -70 °C. Inclusion bodies were prepared by extensively washing bacterial material with TE 50/20.

Table 4: Protocol for expression of bispecific antibodies in insoluble inclusion bodies

Step	Comments
Scrape bacteria into prepared broth.	May start with a seed culture of 50 mL prior to distributing equally among six 2 L flasks.
Incubate at 37 ºC with shaking at 250 rpm until OD600 = 1 to 1.5.	~ 8 hours.
Add IPTG to a final concentration of 1 mM.	
Continue incubation between 90 minutes and 4 hours, but not overnight.	
Centrifuge culture at 3500 rpm for 15 minutes at 4 ºC.	Can freeze pellet after this step or continue with protocol. A freeze/thaw may help with lysis.
Resuspend cells in 810 mL TE50/20 with a	TE50/20: 50 mM Tris, pH 7.5, 20 mM

tissumizer and divide into six 250 mL bottles for a Sorval GSA rotor.	EDTA. Each bottle contains about 135 mL.
Incubate with shaking at room temperature for 30 minutes.	
Add 10 mL (per L) of 5 mg/mL stock solution of lysozyme in H_2O to the resuspension.	
Incubate 30 minutes with shaking.	
Add 15 mL of 5 M NaCl.	
Add 15 mL of 25% Triton X-100.	
Tissumize and incubate for 30 minutes at room temperature with frequent shaking.	
Centrifuge at 11,000 rpm for 50 minutes at 4 °C.	At this step, save the supernatant to ensure that the Triton X-100 solution did not remove the protein of interest.
Resuspend pellet in 1% Triton X-100 in TE50/20 (135 mL per bottle) with a tissuemizer.	Pellet should be a white stripe on the side of the bottle.
Centrifuge at 11,000 rpm for 40 minutes. Discard supernatant.	
Repeat wash three times but using only TE50/20 (no triton).	Can store inclusion bodies at -70 °C or continue on to solubilizing steps.

3.2.3 Solubilizing and Refolding Bispecific Antibodies

IBs were solubilized in 6 M guanidine-HCl, reduced with dithioerythritol, and then refolded by 100-fold dilution into renaturation buffer (0.1 M Tris, 0.5 M arginine-HCl, 0.9 mM oxidized glutathione, 2 mM EDTA, pH 10.3) at 4 °C with rapid mixing, followed by incubation at 4 °C for 72 hours. After renaturation, the solution was dialyzed 3:50 against 0.1 M Tris, 0.5 M NaCl three times and filtered (0.2 μm) to prepare for metal ion affinity chromatography.

Table 5: Protocol for solubilizing and refolding bispecific antibody from insoluble inclusion bodies.

Step	Comments
Resuspend pellet in a solubilization buffer and tissuemize.	In this case, 8 M Guanidine HCl, 100 mM Tris pH 7.5 works well. Start with 10 mL and add more buffer until ~90% solubilized.
Incubate and shake at room temperature for 2 hours.	Can incubate overnight if desired.
Spin down at 15,000-18,000 rpm to pellet unsolubilized inclusion bodies. Save only supernatant.	
Determine protein concentration.	Can be performed with standard Bradford assay technique.
Dilute the protein to 10 mg/mL using solubilizing buffer	
At dithioerythritol to a final concentration of 65 mM (10 mg/mL).	Usually one round of reducing is performed with 300 mg protein.
Prepare 3 L refolding buffer and chill to 4 ºC.	Refolding buffer: 0.1 M Tris pH 8.0, 0.5 M L-Arginine-HCl (105 g/L), 0.9 mM GSSG glutathione, oxidized (0.55 g/L), and 2 mM EDTA.
Adjust the buffer to pH 10.3 by adding NaOH.	~20 g NaOH per L.
Add 10 mL of reduced, solubilized protein to one liter of chilled refolding buffer.	Add protein while buffer is rapidly stirring in a continuous flow of approximately 10 mL per 20 seconds, with a pipette.
Incubate solution at 10 ºC for at least 72 hours.	
Dialyze solution 3 L against 50 L in a buffer suitable for method of purification.	In this case, 0.5 M NaCl, 0.1 M Tris pH 8.0.

3.2.4 Purification of Bispecific Antibodies by Metal Affinity Chromatography

Refolded bispecific antibody was purified by fast protein liquid chromatography (BioLogic DuoFlow 10 System; Bio-Rad) using TALON metal affinity resin (Clontech).

Briefly, sample was loaded by constant controlled flow at 2 mL/min and then the coluμmn was washed with 0.1 M Tris, 0.5 M NaCl until the 280 nm absorbance of the eluate returned to 0. Protein was eluted by stepwise imidazole gradient at a flow rate of 1 mL/min. The product was concentrated with 10,000 MWCO columns (Sartorius Stedim), dialyzed against PBS, and sterile filtered. Concentrations were determined by Bradford assay. The product was subjected to SDS-PAGE analysis (2 μg of total protein per lane) under reducing conditions for purity by silver stain.

3.2.5 Western Blot Analysis

Following manufacturer instructions, proteins were transferred to membranes and incubated with anti-his(C-term) antibody-HRP (Invitrogen) at a 1:5000 dilution in PBS containing 0.05% Tween-20 and 5% nonfat, dry milk. Protein was then detected using SuperSignal West Femto Substrate (Thermo Scientific).

3.2.6 Surface Plasmon Resonance

Binding to antigen was measured by surface plasmon resonance (Karlsson et al., 1991) using the GE Healthcare BIAcore 3000. Antigen was coupled to the surface of carboxylated dextran-coated CM5 research grade chips at pH 4.0 using the amide coupling reagents provided with the BIAcore system. Solutions of bscEGFRvIIIxCD3 (100 nM, 200 nM, and 300 nM) were then passed over the chip to measure binding. Regeneration was performed with 10 mM Glycine pH 2.0. Nonspecific binding was

determined using a flow cell on a chip without antigen. Binding kinetics were analyzed by using BIAcore 3000 software.

3.2.7 *In Vitro* Cytotoxicity

The ability for bscEGFRvIIIxCD3 or nonspecific tandem scFv to redirect cytotoxicity from previously unstimulated human PBMCs was analyzed by standard chromium release assay in round-bottom 96-well plates. Briefly, target cells were labeled with 51Cr and incubated with bscEGFRvIIIxCD3 at 10 μg/mL. PBMCs were then added at effector-to-target (E:T) ratio (20:1) to each well, after which the plates were centrifuged and incubated at 37 °C for 18 hours. The supernatant was then removed and measured by gamma counter.

3.3 Results

3.3.1 Design, Expression and Purification of bscEGFRvIIIxCD3

To construct an EGFRvIII-specific bscAb, we selected an affinity-matured anti-human EGFRvIII-specific scFv, MR1-1 (Kuan et al., 2000), and translated it in tandem with a scFv isolated from the anti-human CD3 monoclonal antibody (mAb), OKT3. Briefly, two scFvs directed against EGFRvIII and CD3, respectively, were joined by a flexible five-amino acid (Gly_4Ser) linker using overlap extension polymerase chain reaction in the following variable domain orientation: V_H-V_L-V_H-V_L.

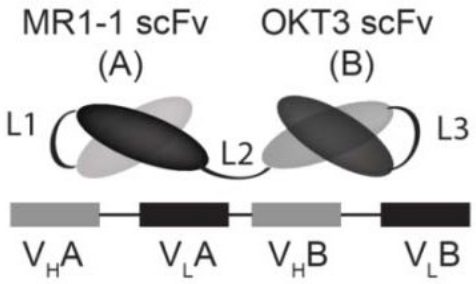


Figure 8: Schematic of variable region sequence of bscEGFRvIIIxCD3.
Schema of bscEGFRvIIIxCD3 design. Linkers L1 and L3 were constructed between the V_H and V_L domains of their respective scFvs and consist of $(Gly_4Ser)_3$, while L2 was constructed as a single Gly_4Ser linker between scFvs.

The resultant tandem single-chain bscAb, bscEGFRvIIIxCD3, was expressed in transformed *E. coli*, isolated from insoluble inclusion bodies and verified for identity by Western blot. Refolded bscAb was purified by metal affinity chromatography with a distinct peak observed in the elution profile between 50 and 100 mM imidazole. Fractions were collected and tested for purity by SDS-PAGE, indicating a single band of refolded bscAb with a predicted molecular weight of approximately 52 kDa. In a typical preparation cycle, 100 mg of inclusion bodies from 1 L of culture yielded 1-2 mg of purified bscEGFRvIIIxCD3.

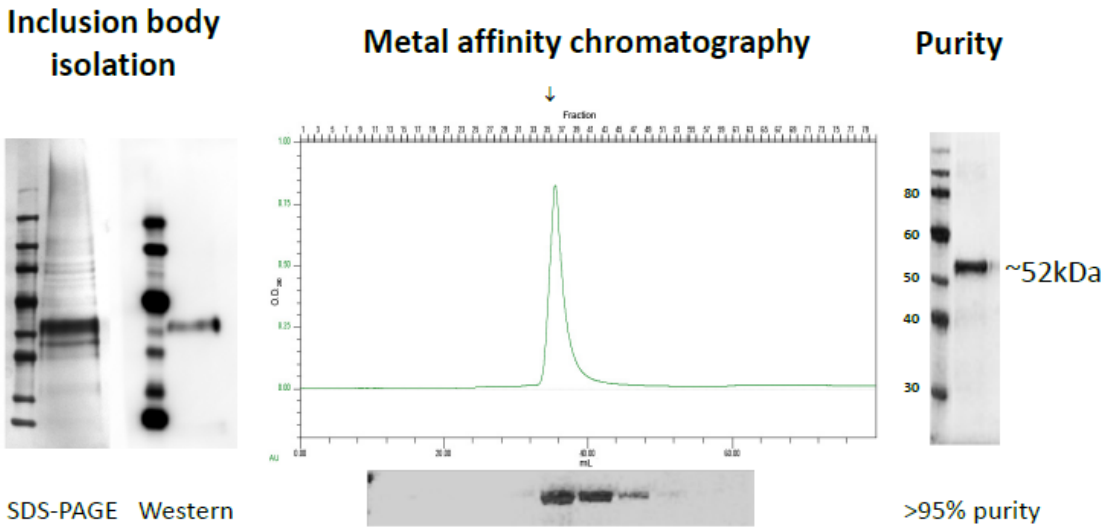


Figure 9: Purification of solubilized inclusion bodies by metal affinity chromatography.

(Left) BscEGFRvIIIxCD3 was expressed as insoluble inclusion bodies and verified for identity by SDS-PAGE and Western Blot with anti-His-tag antibody. (Center) Protein was eluted from an immobilized metal affinity chromatography column by stepwise imidazole gradient. Representative gel from corresponding fractions is shown below. (Right) SDS-PAGE of eluted fraction indicated by arrow in center panel. Molecular weight markers (kDa) are shown on the right.

For the sake of simplicity, we will save discussion regarding further characterization of the EGFRvIII BiTE until the next chapter. The immediate sections that follow will introduce a new method that we have developed to produce negative control, or inert, tandem scFvs. Importantly, these control constructs represent a necessary aspect of our downstream evaluation of the EGFRvIII BiTE and thus warrant detailed discussion here.

3.3.2 Rational Design of Control Reagents for the Evaluation of Bispecific Tandem ScFvs

Properly controlled experiments are essential to the execution of science. As such, a project that seeks to develop a novel biological therapy must also consider the development of parallel control reagents; that is to say, molecules that are carefully designed to be as similar to the active agent as possible, but altered slightly so as to reveal the features of the molecule that are necessary to mediate a given activity of interest—in this case, antitumor efficacy. One way that this concept applies to the field of bispecifics is the production of tandem scFv constructs that maintain specificity for the CD3 T-cell activating complex, yet no longer possess reactivity to surface antigens present on tumor cells. The incorporation of such a molecule as a negative control would allow us to define whether observed antitumor functions were strictly dependent on dual binding specificities, or whether other potential mechanisms were at play that would otherwise suffice for eliciting BiTE-related immune activation.

In order to create a tandem scFv that could serve as a negative control in our experiments, we first identified a monoclonal antibody clone, MOPC-21 (mouse IgG1, κ), with no known binding specificity. MOPC-21 was chosen, particularly because it has been screened for reactivity on a variety of resting, activated, live, and fixed rodent and human tissues, and has been shown to be virtually inert in these settings. In our hands, we were able to verify that indeed, while an anti-EGFRvIII antibody clone, L8A4, bound to glioma cells transfected to express EGFRvIII (U87MG.ΔEGFR), MOPC-21 did not have

appreciable binding on either the parental wild-type expressing U87MG or U87MG.ΔEGFR by flow cytometric analysis.

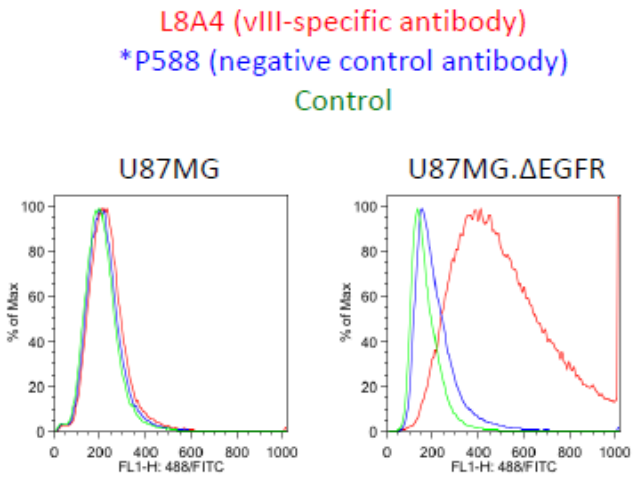


Figure 10: Monoclonal antibody MOPC-21 does not bind to EGFRvIII$^+$ tumor.

Next, we sought to develop a scFv from the MOPC-21 antibody, and translate it in tandem with the OKT3 scFv that we had been previously incorporated into the EGFRvIII BiTE. The intention was to create a tandem scFv that retained the ability to bind CD3 while losing all potential reactivity with target tumor cells.

Unfortunately, we discovered that the new tandem scFv based on the MOPC-21 monoclonal antibody no longer refolded in a conformation that allowed binding to CD3 *via* the OKT3 scFv domain. This effect was most likely due to nuances associated with refolding conditions that we had previously optimized for the EGFRvIII BiTE. Based on the few published attempts at refolding tandem scFvs in this fashion—which were largely unsuccessful (Kipriyanov et al., 2003, Mallender and Voss, 1994, Gruber et al.,

1994)—we theorized that to some degree, each individual protein likely exhibits idiosyncrasies that would prevent achieving identical anti-CD3 bioactivity (*e.g.*, conformation, affinity) through bacterial expression of insoluble inclusion bodies, given the highly variable processes by which non-native proteins refold in solution. Thus, we formulated an alternative method to ablate target cell binding activity while preserving the general refolding properties of bispecific tandem scFv molecules, through limited mutagenesis directed at short portions of complementarity determining regions (CDR).

3.3.2.1 CDR Site-Directed Mutagenesis as a Method to Produce Negative Control Bispecific Antibody Reagents

Since altering one of the two scFvs present in the bispecific molecule was sufficient to abolish native refolding properties, we sought to disrupt binding of the EGFRvIII BiTE to the EGFRvIII tumor antigen through mutations focused on more restricted portions of MR1-1. In order to guide our strategy, we carefully examined previously published protein crystal structures (PDB: 18I8) (Landry et al., 2001) of a closely related scFv, MR1, in complex with the PEPvIII antigen using UCSF Chimera software. Our analysis revealed that three CDR domains appeared to be responsible for primary interactions with PEPvIII residues: CDR 2 and 3 of the variable heavy chain (H2 and H3 of V_H) and CDR3 of the variable light chain (L3 of V_L).

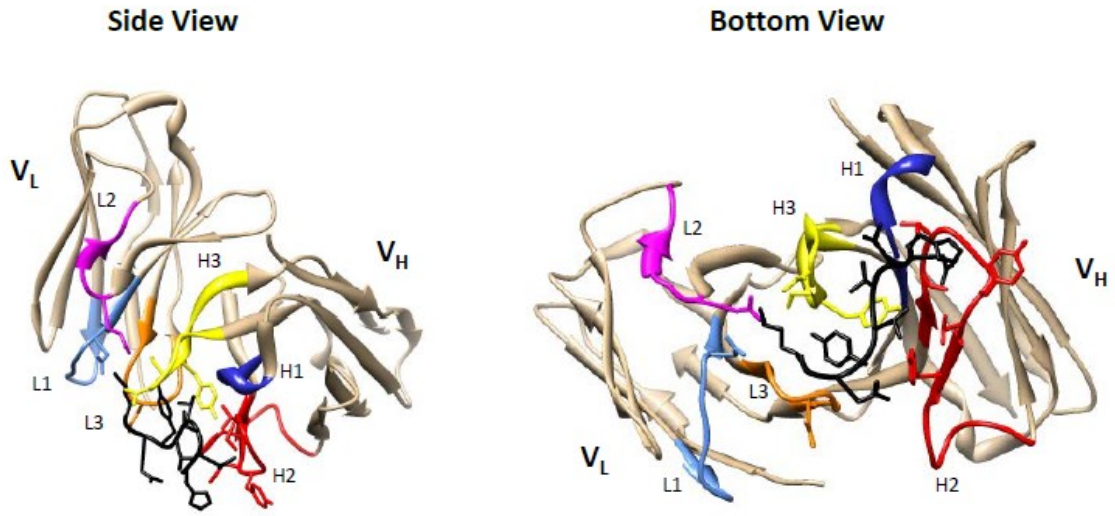


Figure 11: MR1 in complex with PEPvIII (PDB: 18I8).
Side (left panel) and bottom (right panel) views of MR1 in complex with peptide antigen reveals apparent interaction between side chains of the antigen substrate and primarily CDRs H2, H3 and L3.

In addition, UCSF Chimera also provided a quantifiable measure of interactions between side chains of the antigen and the scFv of interest, primarily through approximated measures of intermolecular hydrogen bonds. These data confirm that, among the hydrogen bonds estimated to most influence binding, CDRs corresponding to H2, H3 and L3 were most widely implicated.

Table 6: Hydrogen bonds between EGFRvIII antigen and MR1 CDRs approximated by UCSF Chimera.

Peptide			CDR	Hbond partner			Å
502.C	Lys	N	H3	403.B	Thr	O	3.120
503.C	Gly	N	L3	92.A	Phe	O	2.842
505.C	Tyr	OH	H3	405.B	Tyr	N	3.056
505.C	Tyr	OH	L3	91.A	Ser	O	2.721
506.C	Val	N	H2	359.B	Tyr	OH	2.795
507.C	Val	O	H2	353.B	Thr	N	2.863
508.C	Thr	$O^{\gamma 1}$	H3	402.B	Ser	O	2.757
509.C	Asp	$O^{\delta 2}$	H2	352.B	Ser	O^{γ}	2.832

Based on the above analyses, we sought to perform mutagenesis on the CDRs most likely involved with binding to key residues of the EGFRvIII antigen as determined by UCSF Chimera software. We hypothesized that replacing MR1-1 CDR residues with analogous residues from the MOPC-21 clone scFv, p588, would allow us to abolish EGFRvIII specificity, without disrupting the natural architecture of what could be a biochemically stable CDR domain. Prior to performing the mutagenesis, we aligned the CDRs of the variable heavy and variable light chains of MR1-1 and p588, using conserved residues present in the backbone of each scFv to anchor our analysis.

Variable Heavy Chain

	-CDR1-	-------CDR2-------	----CDR3----
MR1-1 (V_H)	RKFGMS	SISTGGYNTYYSDNVKG	GYSPYSYAMDY
p588 (V_H)	SSFGMH	YISSGSSTLHYADTVKG	WGNYPHYAMDY

Variable Light Chain

	----CDR1----	--CDR2--	---CDR3---
MR1-1 (V_L)	MTSTDIDDDMN	EGNTLRP	LQSWNVPLT
p588 (V_L)	KASENVVTYVS	GASNRYT	GQGYSYPYT

Targeted Mutagenesis

Designation	Replaced Domains	Sequence Homology
H3.H2.L3	CDR3 (V_H), CDR2 (V_H), CDR3 (V_L)	94.4%

Figure 12: Alignment of CDR domains between MR1-1 and p588 variable chains. Disparities between MR1-1 and p588 CDR residues in H2, H3 and L3 are highlighted in red text. Underlined residues indicate those to be replaced with corresponding residues present in the p588 scFv. Targeted mutagenesis as indicated for all three regions would result in an overall sequence homology between the two molecules of greater than 94% (bottom panel).

We next sought to express and purify the mutagenized tandem scFv—which was 94.4% homologous by cDNA sequence with the EGFRvIII BiTE—according to the methods previously optimized for the EGFRvIII BiTE. In line with our predictions, the mutagenized bispecific antibody containing the scFv, H3.H2.L3, behaved similarly to the EGFRvIII BiTE under the same conditions throughout the processes of expression, refolding and purification. Importantly, we were able to purify H3.H2.L3 to a single band of an expected molecular weight, approximately 52 kDa.

Figure 13: SDS-PAGE of H3.H2.L3 demonstrates highly pure protein product. Molecular weight ladder is listed on the left demonstrates purification of a protein approximately 52 kDa.

When compared with EGFRvIII BiTE, the mutant H3.H2.L3 did not bind at detectable levels when tested against EGFRvIII-expressing tumors by flow cytometric analysis. However, H3.H2.L3 did continue to bind the CD3 surface antigen on both $CD4^+$ and $CD8^+$ T cells, which were stained as a fraction of human PBMC. These data were the first evidence that demonstrated our successful production of an adequate control bispecific tandem scFv with high sequence homology to the original EGFRvIII.

In addition, we employed surface plasmon resonance to quantify whether there were any significant differences with regard to CD3 bioactivity between the EGFRvIII BiTE and the tandem scFv mutant that may have been beneath the limit of detection of flow cytometric analysis. In our analysis, while EGFRvIII BiTE bound as expected to both EGFRvIII extracellular domain and to CD3ε, the H3.H2.L3 mutant demonstrated

no detectable binding (NDB) to EGFRvIII antigen, while maintaining virtually indistinguishable kinetics from the EGFRvIII BiTE with regard to CD3ε.

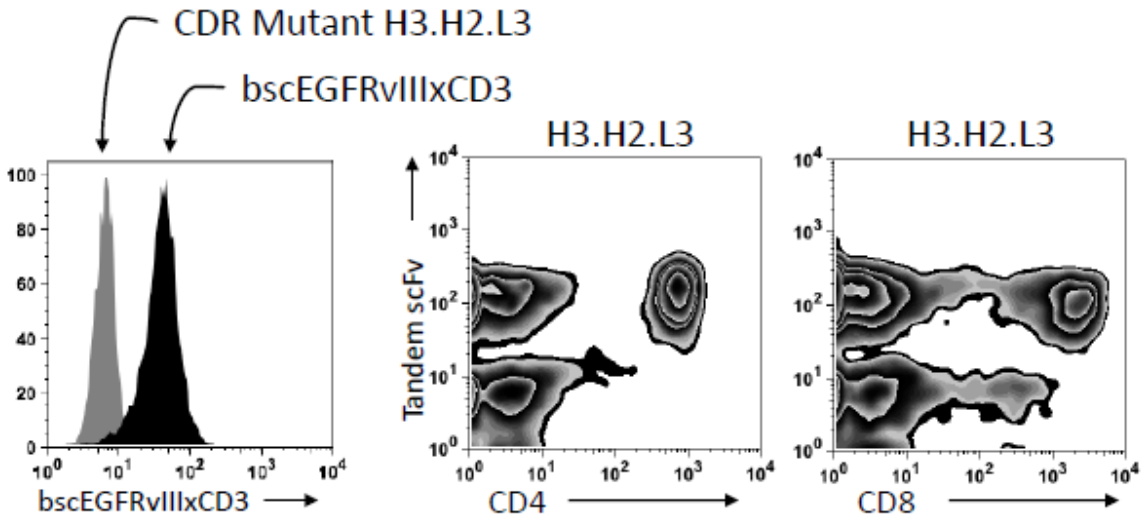


Figure 14: The H3.H2.L3 mutation ablates EGFRvIII binding while maintaining specificity for CD3 by flow cytometry.
H3.H2.L3 no longer binds to tumors expressing EGFRvIII (U87MG.ΔEGFR) (Left Panel), but continues to bind to T cells that coexpress surface CD4 and CD8 (Middle and Right Panels, respectively).

Table 7: The H3.H2.L3 mutation demonstrates no detectable binding to EGFRvIII by surface plasmon resonance but continues to bind CD3 at expected levels.

	K_d (M)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_d (M)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})
EGFRvIII	1.5×10^{-9}	4.6×10^{4}	8.1×10^{-4}	NDB	NDB	NDB
CD3	6.5×10^{-9}	6.61×10^{4}	4.29×10^{-4}	5.6×10^{-9}	3.2×10^{4}	1.8×10^{4}
	EGFRvIII BiTE			**H3.H2.L3**		

Lastly, based on the well-characterized capacity of antibodies to recognize a vast array of antigenic substrates through relatively minor permutations of V(D)J recombination, we hypothesized that even single CDR mutations would be sufficient to

significantly abrogate EGFRvIII binding and thus antitumor efficacy of the EGFRvIII BiTE. Towards this end, we created three additional tandem scFvs, one of each with individual H3, H2 or L3 mutant CDRs, again corresponding to residues from the nonspecific scFv, p588. As expected, we were able to refold and purify all gene products following site-directed mutagenesis, without having to otherwise alter our methodology. The highest sequence homology achieved was 98.8%, which reflected a change of six amino acids in H3, a relatively small portion of the entire tandem scFv.

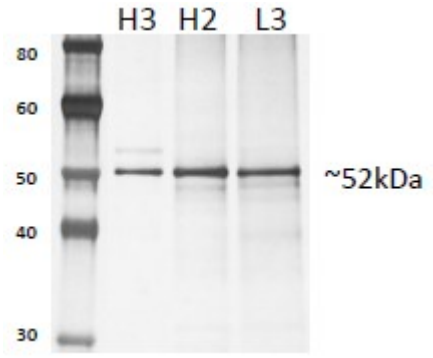


Figure 15: SDS-PAGE demonstrates that single CDR mutant tandem scFvs can be purified with high fidelity to create negative control tandem scFvs.
H3, H2 and L3 single CDR mutants are purified by metal affinity chromatography and are represented here as a band approximately 52 kDa. Molecular weight markers are shown on the left.

Table 8: Sequence homology among all mutant tandem scFv included in the study.

	Targeted Mutagenesis	
Designation	**Replaced Domains**	**Sequence Homology**
H3.H2.L3	CDR3 (V_H), CDR2 (V_H), CDR3 (V_L)	94.4%
H3	CDR3 (V_H)	98.8%
H2	CDR2 (V_H)	97.2%
L3	CDR3 (V_L)	98.4%

Importantly, we not only sought to abrogate the capacity of mutant tandem scFvs to bind EGFRvIII by measures of flow cytometry and surface plasmon resonance, but also performed functional tests to evaluate whether EGFRvIII-targeted BiTEs would mediate immune responses against tumors expressing EGFRvIII, and whether this activity would be dependent on dual binding specificity *in vivo* (as demonstrated by absence of cytotoxicity in the presence of the negative control molecule). In a standard chromium release assay, EGFRvIII BiTE was shown to mediate specific lysis against EGFRvIII-expressing glioma *in vitro.* Here, the mutated tandem scFvs—which all retained the ability to bind CD3—were unable to redirect a similar response against EGFRvIII-expressing cells.

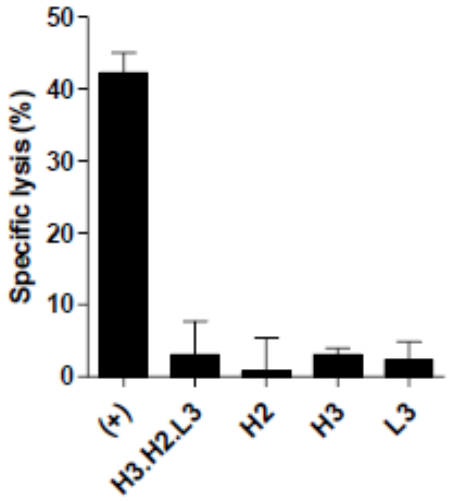


Figure 16: Single CDR mutagenized tandem scFvs can serve as highly homologous, negative control molecules for the preclinical evaluation of BiTEs.
Standard ^{51}Cr-release demonstrates specific lysis of U87MG.ΔEGFR by unstimulated human PBMCs and bscEGFRvIIIxCD3 (+), as well as four other mutagenized constructs meant to serve as negative controls (E:T ratio 20:1; incubation time 18 hours; [tandem scFv] 10 μg/mL

This finding and other aspects of characterization regarding BiTE mediated antitumor activity will be the central premise of the chapter that follows.

4 Systemic Administration of a Bispecific Antibody Targeting EGFRvIII Successfully Treats Intracerebral Glioma

4.1 Introduction

Bispecific antibodies (bscAbs), particularly those of the bispecific T-cell engager (BiTE) subclass, have been shown to effectively redirect T cells against cancer.

To date, various mouse models and clinical trials have demonstrated the great potential of antibody-redirected T-cell therapies, either through chimeric antigen receptors (CARs) or more recently *via* BiTEs against tumor-associated antigens including ErbB-2, CEA, and CD19 (Baeuerle and Reinhardt, 2009, Brentjens et al., 2010, Morgan et al., 2010, Klinger et al., 2012). However, because these targets are not strictly limited to tumor tissue, such approaches have led to unwanted toxicity and destruction of even normal, healthy cells (Bargou et al., 2008, Heslop, 2010). Moreover, like other large molecules, bscAbs may be restricted from entry into the "immunologically-privileged" CNS.

In previous chapters, we have discussed in detail the several advantages of therapeutically targeting the EGFRvIII tumor-specific antigen. EGFRvIII is a constitutively-activated tyrosine kinase not found in normal tissues, but frequently expressed in GBM and many other neoplasms. Because it is solely localized to tumor tissue, EGFRvIII represents an ideal target for immunotherapy.

Here, we describe the preclinical evaluation of the EGFRvIII-targeted BiTE, bscEGFRvIIIxCD3, the design and manufacture of which was detailed in the preceding chapter. Importantly, our results show that bscEGFRvIIIxCD3 activates T cells to mediate potent and antigen-specific lysis of EGFRvIII-expressing glioma *in vitro* ($p<.001$) at exceedingly low concentrations (10 ng/mL) and effector-to-target ratios (2.5:1). Intravenous treatment with bscEGFRvIIIxCD3 led to extended survival in mice with well-established intracerebral tumors ($p<.05$) and achieved durable complete cures at rates up to 75%. Antitumor efficacy was significantly abrogated upon blockade of EGFRvIII-binding, demonstrating the need for target-antigen specificity both *in vitro* and *in vivo*.

These results demonstrate for the first time that BiTEs can be used to elicit functional antitumor immunity in the CNS. Moreover, peptide blockade of BiTE-mediated activity is shown to be a novel strategy that may greatly enhance the safety profile for antibody-redirected T-cell therapies. Finally, bscEGFRvIIIxCD3 represents a unique advance in BiTE technology given its exquisite tumor-specificity, which enables precise elimination of cancer without the risk of autoimmune toxicity.

4.2 Materials and Methods

4.2.1 Mice and Tumor Cell Lines

NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ, also known as NOD scid gamma (NSG) mice, were purchased from The Jackson Laboratory and were bred under pathogen-free

conditions at Duke University. All animal experiments were performed according to protocols approved by the Duke University Institutional Animal Care and Use Committee. The human glioma cell lines U87MG and D54, as well as their respective sublines, U87MG.ΔEGFR and D54-EGFRvIII have been previously described (Nishikawa et al., 1994, Lal et al., 2002).

4.2.2 Flow Cytometric Analysis

Lymphocytes were incubated for 15 minutes at room temperature with combinations of titrated antibodies to CD4 (RPA-T4), CD8 (RPA-T8), CD25 (M-A251) and CD69 (L78). Cells were washed and analyzed on a BD Biosciences FACSCalibur flow cytometer. Data analysis was performed using BD FloJo software. In certain experiments, bscEGFRvIIIxCD3 was added to lymphocytes or tumor cells at a concentration of 10 μg/mL and allowed to incubate for 15 minutes at room temperature. Cells were washed and incubated with Penta-His Alexa Fluor 488 Conjugate (Qiagen) and analyzed as above.

4.2.3 Proliferation Assay

In experiments assessing proliferation, 1 μCi ^{3}H-thymidine was added to each well of a 96-well plate. Cells were cultured for an additional 24 hours and collected by a cell harvester. Counts were performed using a Wallac 1450 Microbeta Trilux Liquid

Scintillation/Luminescence Counter (Perkin-Elmer) and data were recorded as means of triplicate wells.

4.2.4 T-cell Activation and Cytometric Bead Array

Freshly thawed PBMCs were placed in 96-well plates with bscEGFRvIIIxCD3 (10 μg/mL) and target cells at an E:T ratio of 20:1 in a total volume of 200 μL. Supernatant was removed after incubation for 48 hours at 37 °C and analyzed by manufacturer's instructions. Cells were also harvested and analyzed for activation markers as above.

4.2.5 *In vitro* Cytotoxicity

The ability for bscEGFRvIIIxCD3 to redirect cytotoxicity from previously unstimulated human PBMCs was analyzed by standard chromium release assay in round-bottom 96-well plates. Briefly, target cells were labeled with ^{51}Cr and incubated with bscEGFRvIIIxCD3 at varying concentrations. PBMCs were then added at various effector-to-target (E:T) ratios (ranging from 1:1 to 20:1) to each well, after which the plates were centrifuged and incubated at 37 °C for 18 hours. The supernatant was then removed and measured by gamma counter. Where noted, peptide blockade was performed with the addition of PEPvIII to wells at a 1:1 ratio by mass with bscEGFRvIIIxCD3. In certain experiments, target cells were labeled using PKH26 red fluorescent cell linker kit (Sigma) and effector cells were labeled using intracellular carboxyfluorescein diacetate succinimidyl ester (CFSE) according to manufacturer

instructions. Cells were then visualized across multiple time points using a Nikon Eclipse TE2000-E fluorescent microscope.

4.2.6 Tumor Implantation

U87MG and U87MG.ΔEGFR were grown in improved MEM zinc option media with 10% FBS. Tumor cells were collected in logarithmic growth phase, washed twice with PBS, and mixed with an equal volume of 10% methyl cellulose prior to loading into a 250 μL syringe with an attached 25 gauge needle. The needle was positioned using a stereotactic frame at 2 mm to the right of the bregma and 4mm below the surface of the skull at the coronal suture. The tumorigenic dose was 1 x 10^5 cells in a total volume of 5 μL. For certain experiments, freshly thawed human PBMCs were resuspended with tumor cells prior to inoculation at a ratio of 1:1. Treatment with bscEGFRvIIIxCD3 was infused in a total volume of 250 μL by tail vein injection on the indicated days following tumor implantation. Where noted, peptide blockade was performed with the co-infusion of soluble PEPvIII by tail vein at a 1:1 ratio with bscEGFRvIIIxCD3.

4.2.7 Statistical Analysis

Unless otherwise stated, groups were compared using a two-sample two-tailed t-test to determine statistical significance. Survival curves were estimated for each group using the product-limit estimation of Kaplan and Meier. Primary comparative analyses

of the curves for each group were performed using the generalized Wilcoxon test. Statistical significance was determined at a value of $P < 0.05$.

4.3 Results

4.3.1 BscEGFRIIIxCD3 Redirects T cells Against Tumor Cells *in Vitro*

First, we sought to test bscEGFRvIIIxCD3 for its ability to activate T cells *in vitro*. When incubated with bscEGFRvIIIxCD3 and target cells, $CD4^+$ and $CD8^+$ T cells upregulated surface expression levels of activation markers CD69 and CD25. Importantly, this required interaction with the EGFRvIII antigen, since elevated CD69 and CD25 expression was detected only in the presence of EGFRvIII-expressing human glioma cells compared to wild-type parental controls.

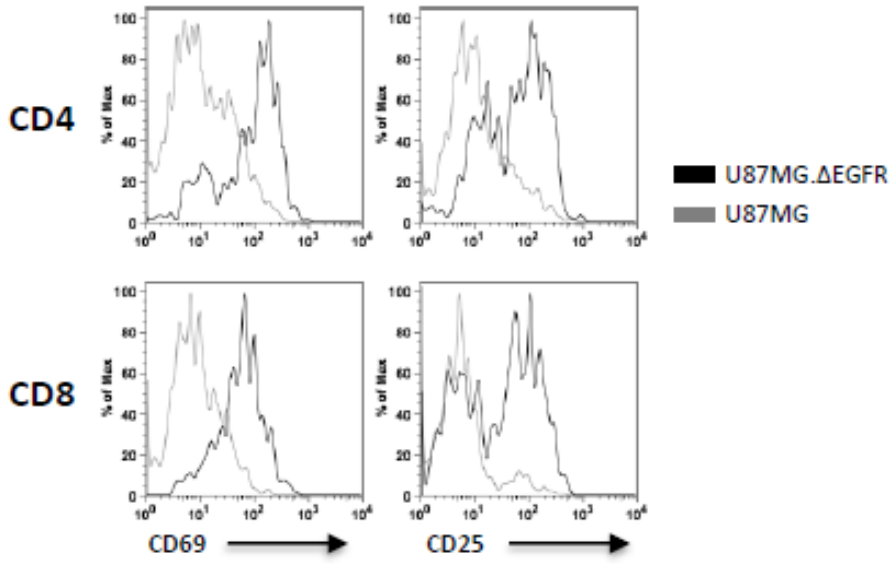


Figure 17: Expression of activation markers on T cells redirected by bscEGFRvIIIxCD3.
T cells incubated with bscEGFRvIIIxCD3 and either U87MG.ΔEGFR (black line) or U87MG (grey line) were stained and analyzed for surface expression of activation markers CD69 and CD25.

The ability of bscEGFRvIIIxCD3 to induce antigen-specific proliferation of T cells was also measured by ^{3}H-thymidine incorporation *in vitro*. In these experiments, recombinant EGFRvIII extracellular domain (ECD) was used as a source of cognate antigen in order to remove the variability in ^{3}H-thymidine incorporation that would otherwise occur due to proliferation of target glioma cells. When soluble bscEGFRvIIIxCD3 was incubated in the presence or absence of solid-phase bovine serum albumin (BSA), proliferative effects were not detected above baseline levels. However, soluble bscEGFRvIIIxCD3 in the presence of solid-phase EGFRvIII ECD led to

significant proliferation that was indistinguishable from stimulation with solid-phase bscEGFRvIIIxCD3 alone. This verified that—as has been well-characterized for BiTEs against other antigens—interaction between soluble bscEGFRvIIIxCD3 and the EGFRvIII target is necessary in order to potentiate immunological capping and synapse formation, and that antigen-specificity represents a crucial factor mediating this activity (Offner et al., 2006).

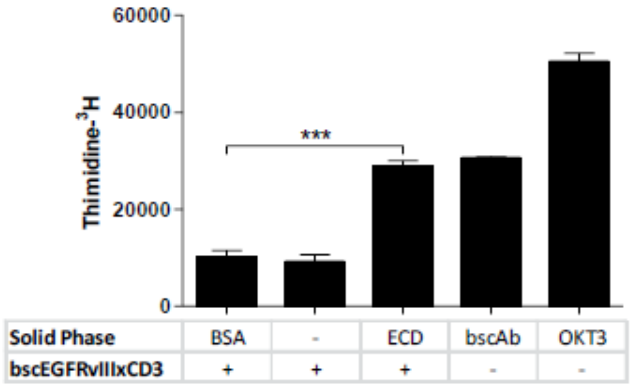


Figure 18: BiTE-redirected T cells proliferate in the presence of EGFRvIII. Proliferation of T cells in response to bscEGFRvIIIxCD3 and solid phase EGFRvIII ECD was measured by ^{3}H-thymidine incorporation. *** $P < 0.001$.

Effective antitumor immune responses are known to require the secretion of inflammatory cytokines, particularly those associated with T_H1 polarization. To test whether activation of T cells by bscEGFRvIIIxCD3 leads to favorable cytokine production, cytometric bead array was used to analyze the supernatants of cultures in which lymphocytes were incubated with bscEGFRvIIIxCD3 and target cells either

expressing or not expressing the EGFRvIII tumor-specific antigen. In the presence of negative control target cells, analyses of culture supernatants revealed minimal secretion of IL-2, IFN-γ and TNF. However, when incubated with EGFRvIII-expressing glioma, bscEGFRvIIIxCD3 elicited significantly greater T-cell function, which, when represented as proportions in a multiplex cytokine panel, demonstrated considerable polarization toward a T_H1-associated immune response.

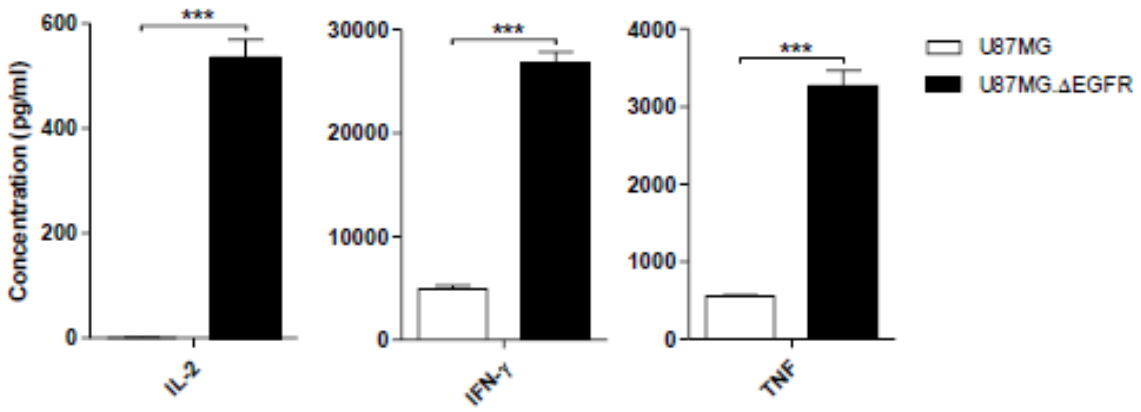


Figure 19: CBA anlaysis of supernatants from BiTE-redirected T cells *in vitro*. Supernatants from wells containing unstimulated human PBMCs with bscEGFRvIIIxCD3 and either U87MG.ΔEGFR or U87MG were subjected to CBA analysis for inflammatory cytokines, demonstrating elevated secretion of IL-2, IFN-γ, and TNF in the presence of U87MG.ΔEGFR. *** $P < 0.001$.

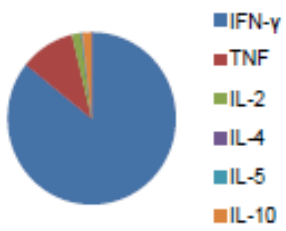


Figure 20: EGFRvIII BiTEs elicit Th1 polarized cytokine secretion.

Next, we sought to test bscEGFRvIIIxCD3 for its ability to functionally elicit antigen-specific cytotoxic responses *in vitro*. In a standard ^{51}Cr release assay using freshly-thawed human PBMCs as effector cells, we determined that bscEGFRvIIIxCD3-mediated redirection of T cells is indeed highly cytotoxic against multiple previously characterized human EGFRvIII-expressing tumor cells (Lal et al., 2002).

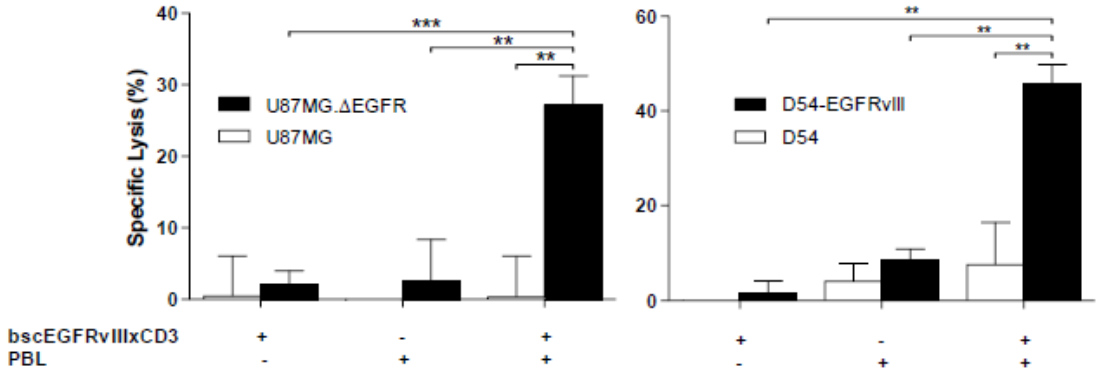


Figure 21: BiTEs redirect T cells to kill EGFRvIII tumor *in vitro*.
Standard ^{51}Cr-release demonstrates specific lysis of U87MG.ΔEGFR over U87MG (left panel) and D54-EGFRvIII over D54 (right panel) by unstimulated human PBMCs and bscEGFRvIIIxCD3 (E:T ratio 20:1; incubation time 18 hours; [bscEGFRvIIIxCD3] 10 μg/mL). *** $P < 0.001$, ** $P < 0.01$.

Importantly, cytotoxicity was strictly dependent on the ability for bscEGFRvIIIxCD3 to recognize and bind to the EGFRvIII antigen on target cells, since incubation of bscEGFRvIIIxCD3 with matched EGFRvIII-negative tumors in the presence of effector cells did not induce observable target-cell lysis. Furthermore, demonstrating the need for dual specificity within the actual therapeutic molecule, target-cell lysis was not observed upon incubation with a nonspecific bscAb control bscAbxCD3—which was designed from antigen-binding portions isolated from isotype clone MOPC-21 and OKT3, as detailed in the previous chapter—but was maintained against EGFRvIII-expressing cells upon incubation with the active molecule, bscEGFRvIIIxCD3.

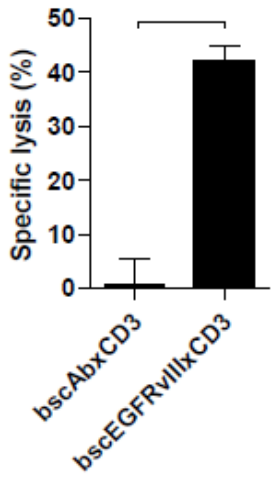


Figure 22: Cytotoxic effects mediated by EGFRvIII BiTEs depend on dual specificity.

Specific lysis of the U87MG.ΔEGFR cell line is not observed in the presence of negative control bscAbxCD3 compared to incubation with bscEGFRvIIIxCD3 (E:T ratio 20:1; incubation time 18 hours; [bscEGFRvIIIxCD3] 10 μg/mL).

In addition to being highly specific, the bioactivity of bscEGFRvIIIxCD3 was also potent against EGFRvIII-expressing tumor at greatly reduced concentrations, with dose-dependence observed at E:T ratios as low as 2.5:1.

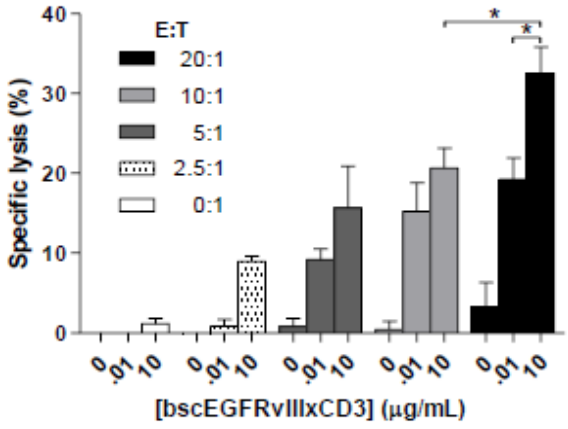


Figure 23: The effects of bscEGFRvIIIxCD3 are potent and dose dependent *in vitro*. Specific lysis of the U87MG.ΔEGFR cell line varies proportionally with bscEGFRvIIIxCD3 concentration and dose response is maintained over varied E:T ratios. * $P < 0.05$.

When visualized by light microscopy, bscEGFRvIIIxCD3 did not appear to affect the behavior of lymphocytes against EGFRvIII-negative glioma cells *in vitro*. However, in the presence of EGFRvIII expression on the surface of matched glioma, lymphocytes visibly localized to the surface of tumor cells upon addition of bscEGFRvIIIxCD3,

implying that the cytotoxic effects observed *in vitro* may occur *via* contact-mediated mechanisms.

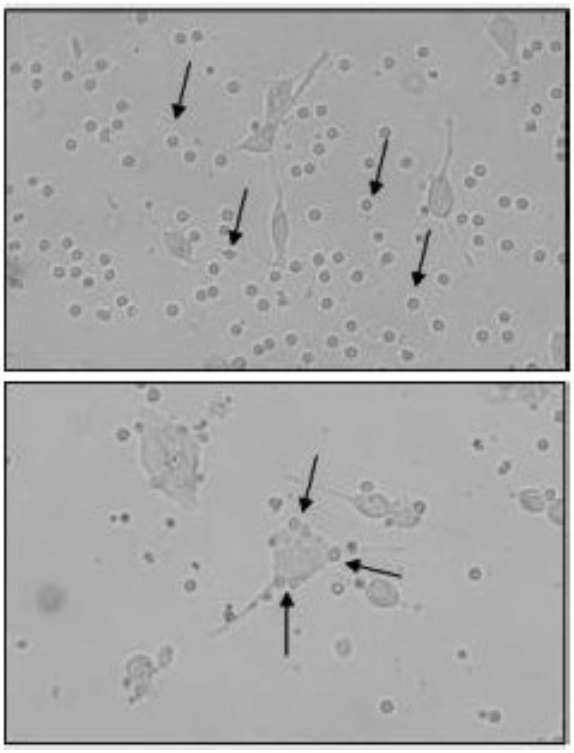


Figure 24: Antigen-specific co-localization of lymphocytes and tumor cells in the presence of bscEGFRvIIIxCD3 *in vitro*.
Light microscopy of T cells incubated with U87MG in the presence of bscEGFRvIIIxCD3 does not demonstrate appreciable localization to tumor (top panel) compared to wells in which target cells expressing EGFRvIII, U87MGΔEGFR, were included (bottom panel).

Importantly, under conditions wherein PKH26-labeled EGFRvIII-positive (red) and matched EGFRvIII-negative (bright field) glioma cells were cultured together in close proximity, bscEGFRvIIIxCD3-redirected CFSE-labeled T cells (yellow) were seen to localize only to EGFRvIII-expressing tumor. These results lend further credence to the

ability of the BiTE therapeutic platform to selectively distinguish between even closely-associated, adjacent cells based on target antigen expression *in vitro*.

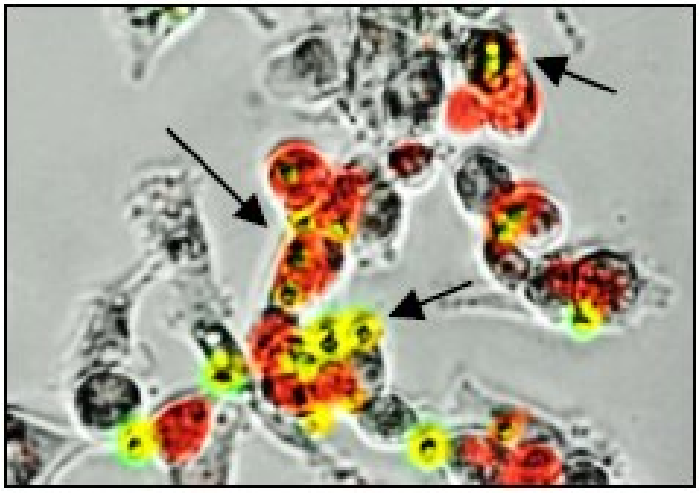


Figure 25: Co-culture of fluorescently labeled lymphocytes and target tumor cell lines +/- EGFRvIII expression *in vitro*.
Experiments were also performed where U87MG (bright field), labeled U87MG.ΔEGFR (red) and T cells (yellow) were incubated with bscEGFRvIIIxCD3, which demonstrated selective localization of T cells to EGFRvIII-expressing tumor cells *in vitro*.

4.3.2 The Antitumor Response Produced by BscEGFRvIIIxCD3 is Specific to EGFRvIII-Expressing Tumors *in Vivo*

To investigate the *in vivo* activity of bscEGFRvIIIxCD3, we established orthotopic xenograft models using the human malignant glioma cell lines U87MG and U87MG.ΔEGFR. A number of studies have recently demonstrated that GBMs are infiltrated by immune cells (Yang et al., 2010, Lohr et al., 2011); thus, in order to model this scenario preclinically, NSG mice were inoculated intracranially with a mixture of tumor cells and unstimulated human PBMCs. Following implantation,

bscEGFRvIIIxCD3 was administered *via* daily tail vein injections. Under these conditions, we observed that mice implanted with tumors expressing only the wild-type EGFR did not exhibit a significant survival benefit. Similarly, in mice implanted with intracerebral tumors expressing EGFRvIII, treatment with the nonspecific control bscAbxCD3 did not prolong survival over mice receiving vehicle control.

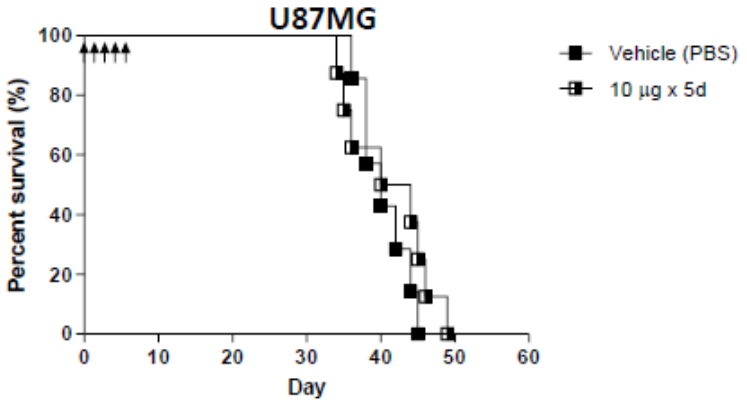


Figure 26: EGFRvIII BiTE does not treat EGFRvIII-negative tumors *in vivo*. NSG mice were implanted intracerebrally with $1x10^5$ tumor cells and unstimulated human PBMCs at a ratio of 1:1. Mice implanted with U87MG were treated with bscEGFRvIIIxCD3 by daily intravenous infusion (arrows).

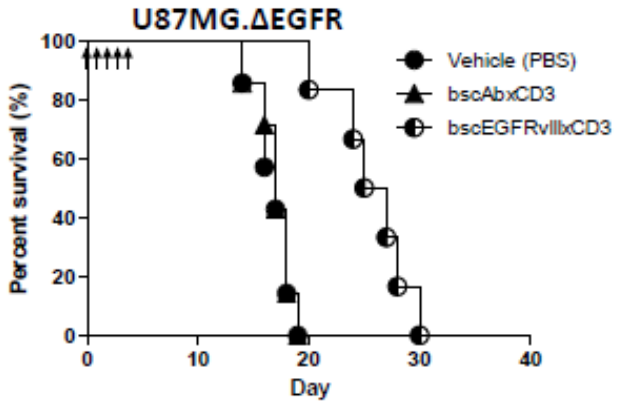


Figure 27: *In vivo* efficacy of the EGFRvIII BiTE is specific.
NSG mice (n=8) were implanted intracerebrally with $1x10^5$ tumor cells and unstimulated human PBMCs at a ratio of 1:1. Mice implanted with U87MG.ΔEGFR were treated with bscEGFRvIIIxCD3 or control bscAbxCD3 by daily intravenous infusion (arrows).

However, infusion with bscEGFRvIIIxCD3 in this setting achieved durable, complete cures in 6/8 mice without apparent toxicity; this effect was also potent and dose-dependent, yielding prolonged survival at doses as low as 1 μg/mouse/day, with comparable results observed in repeated experiments .

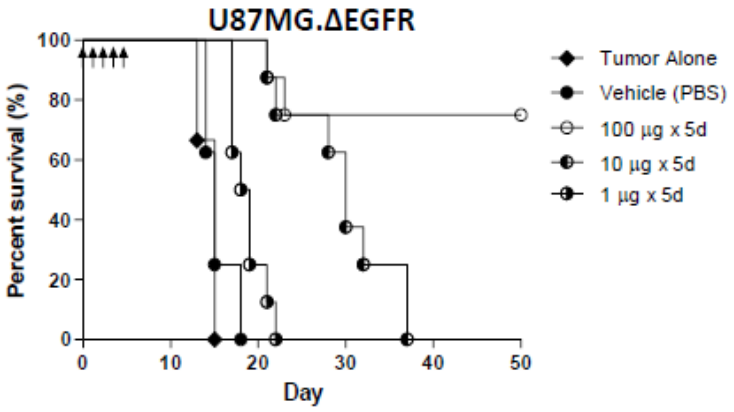


Figure 28: The antitumor activity of bscEGFRvIIIxCD3 against EGFRvIII-expressing tumor is potent and dose-dependent *in vivo*.
Mice implanted with U87MG.ΔEGFR were treated with bscEGFRvIIIxCD3 by daily intravenous infusion (arrows). To assess dose response, bscEGFRvIIIxCD3 was administered to mice at indicated doses.

4.3.3 Antitumor Efficacy of BscEGFRvIIIxCD3 is Abrogated by EGFRvIII Blockade.

Despite early evidence of success in the clinic, recent antibody-redirected T-cell platforms have been shown to induce lethal "on-target" toxicity when their cognate antigen is not only present in tumors but also expressed in normal, healthy tissues (*e.g.*, ErbB-2, CEA and CD19) (Baeuerle and Reinhardt, 2009, Brentjens et al., 2010, Morgan et al., 2010, Klinger et al., 2012). To address this issue in our model—and to provide additional controls demonstrating the specificity of our approach—we sought to determine whether competitive inhibition of EGFRvIII-binding could directly abrogate biological activity of bscEGFRvIIIxCD3 both *in vitro* and *in vivo*. The rationale for this hypothesis was based on our data showing that bscEGFRvIIIxCD3 is not effective in the

absence of EGFRvIII-expression on tumors, and that similarly, a control bscAbxCD3 is not effective in the presence of EGFRvIII-expressing tumors; these data demonstrated that the anti-CD3 moiety alone does not mediate direct antitumor effects. Importantly, our inclusion of selective EGFRvIII-blockade here also provides an additional control that further demonstrates the exquisite specificity of the BiTE platform.

To perform specific EGFRvIII blockade, we employed a previously-published soluble peptide (PEPvIII) corresponding to the extracellular epitope for EGFRvIII-specific antibodies (Heimberger et al., 2003). Importantly, this peptide is currently in clinical trials as an EGFRvIII-targeted vaccine, and thus possesses great translational potential in addition to providing biologic principle. Using this approach, our data demonstrate that EGFRvIII blockade by PEPvIII successfully disrupts binding to EGFRvIII-expressing tumor targets *in vitro*; importantly, PEPvIII inhibition was specific since peptide-blocked bscEGFRvIIIxCD3 retained the ability to bind the CD3 complex on both $CD4^+$ and $CD8^+$ T cells.

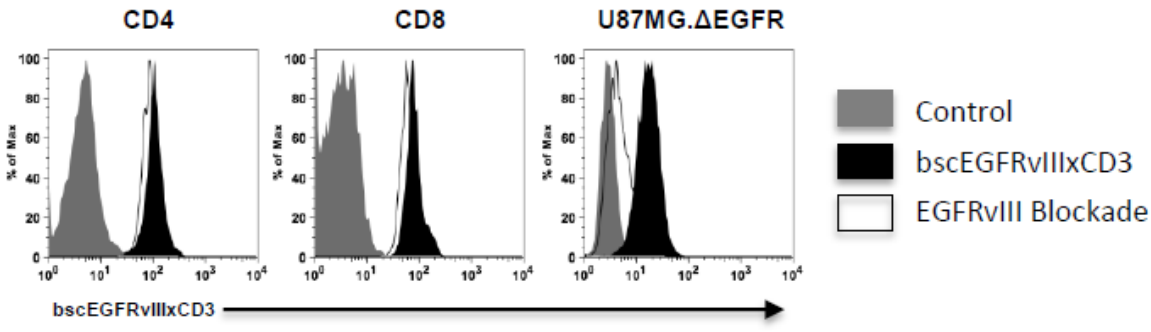


Figure 29: PEPvIII peptide blockade abrogates binding to tumor cells expressing EGFRvIII.

EGFRvIII blockade does not impact the ability for bscEGFRvIIIxCD3 to bind $CD4^+$ or $CD8^+$ T cells, but significantly abrogates binding to EGFRvIII-expressing glioma *in vitro*.

Despite continued anti-CD3 binding, addition of the cognate PEPvIII peptide was sufficient to completely abolish bscEGFRvIIIxCD3-mediated antitumor effects against EGFRvIII-expressing glioma *in vitro*. Moreover, EGFRvIII blockade significantly reduced efficacy of bscEGFRvIIIxCD3 *in vivo* against EGFRvIII-expressing tumor in the brain.

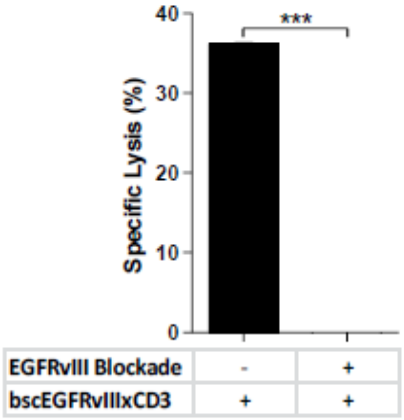


Figure 30: Blockade of BiTE-mediated antitumor activity *in vitro* with EGFRvIII peptide blockade.
Standard ^{51}Cr-release demonstrates specific lysis of U87MG.ΔEGFR in the presence of bscEGFRvIIIxCD3 and effector cells (E:T ratio 20:1; incubation time 18 hours; [bscEGFRvIIIxCD3] 10 μg/mL) which is completely inhibited in the presence of soluble PEPvIII cognate peptide (blockade ratio 1:1). *** $P < 0.001$.

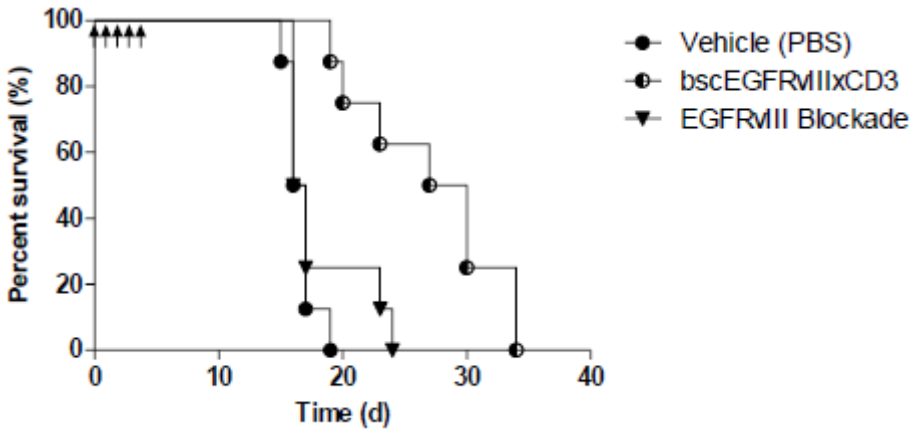


Figure 31: Antitumor efficacy of EGFRvIII BiTE is abrogated upon PEPvIII blockade *in vivo*.
NSG mice (n=8) were implanted i.c. with $1x10^5$ U87MG.ΔEGFR and unstimulated human PBMCs at a ratio of 1:1. Daily intravenous infusions with bscEGFRvIIIxCD3, co-infusion with PEPVIII blockade, or PBS vehicle control began 3 hours after implantation and continued for 5 days (arrows).

Together, these results validate the precise target-specificity of the bscEGFRvIIIxCD3 construct and definitively establish the need for dual-binding given that, upon EGFRvIII blockade, effector-arm interactions with CD3 alone were not sufficient to achieve antitumor activity. In addition, our finding that a soluble cognate peptide can be used to selectively inhibit therapeutic effects of bscEGFRvIIIxCD3 may offer a novel approach to enhancing the safety profile of other bscAbs that target antigens expressed in normal, healthy tissues.

4.3.4 BscEGFRvIIIxCD3 Recruits Circulating Immune Cells to Treat Intracerebral Tumors

Because bscEGFRvIIIxCD3 proved to potently eliminate intracerebral glioma in the presence of even small numbers effector cells, we investigated whether treatment with an EGFRvIII-specific bscAb would lead to the recruitment and accumulation of circulating peripheral lymphocytes to EGFRvIII-expressing glioma cells in the brain. To explore this possibility, tumor cells alone were implanted intracerebrally in mice and allowed to establish for 10 days. Animals were then immune-reconstituted by i.p. infusion with human PBMCs and treated with bscEGFRvIIIxCD3 by tail vein in the same manner as previously described. Tumors examined from mice receiving vehicle (PBS) did not exhibit visible immune infiltrate upon histological analysis and instead showed invasive tumor tissue extending along peritumoral blood vessels . However, in mice receiving bscEGFRvIIIxCD3, peripherally-infused PBMCs were observed to leave peritumoral vessels and traffic toward EGFRvIII-expressing tumor within the brain.

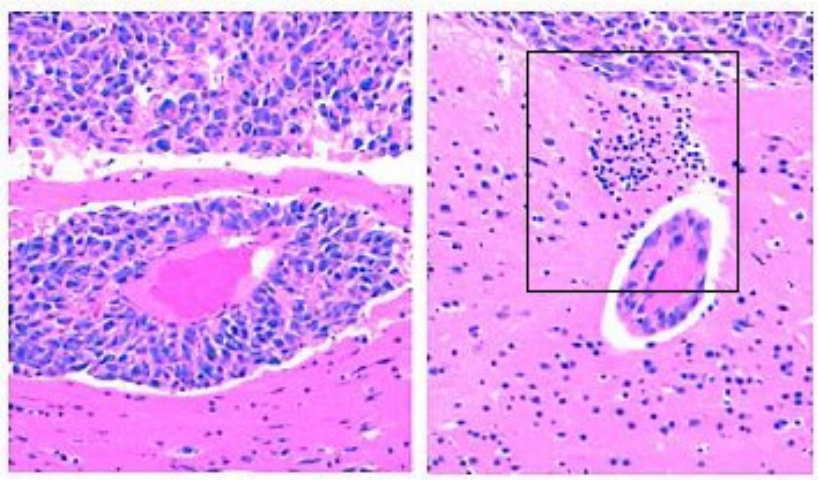


Figure 32: Perivascular lymphocytic cuffing is associated with intracerebral vessels in mice treated with EGFRvIII BiTE.

NSG mice (n=5) were implanted intracerebrally with $1x10^5$ U87MG.ΔEGFR. On Day 10 following tumor implantation, animals were immune-reconstituted peripherally with $2x10^7$ unstimulated human PBMCs by intraperitoneal injection. Daily intravenous infusions with bscEGFRvIIIxCD3 (10 μg/mouse/day) or PBS began on day 10 and were continued for 5 days after which mice were sacrificed and processed for histological sectioning. (Left) Representative section of intracerebral tumor and local invasion surrounding an adjacent vessel in an animal receiving PBS. (Right) By contrast, in mice treated with bscEGFRvIIIxCD3, PBMCs are seen to exit peritumoral vessels toward tumor tissue (box).

Moreover, areas of residual EGFRvIII-expressing tumor in treated mice revealed heavy mononuclear infiltrate and regions of necrotic tumor.

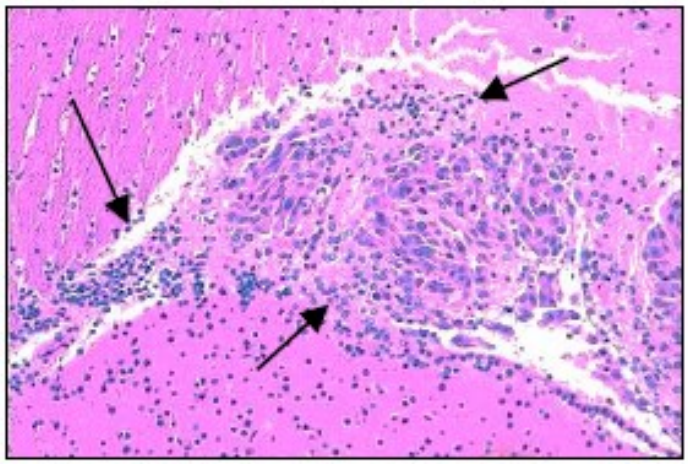


Figure 33: Representative section of residual tumor in treated mice.
Tumor tissue in mice treated with bscEGFRvIIIxCD3 and receiving intraperitoneal PBMCs shows diffuse tumor-associated mononuclear infiltrate and areas of necrosis.

Due to the dramatic impact of bscEGFRvIIIxCD3 on intracerebral localization of cellular immunity, we reasoned that this effect might also be therapeutic against late-stage, established tumors. To test this, animals were peripherally immune-reconstituted and treated with bscEGFRvIIIxCD3 just 5 days before the median survival of mice under control conditions. Our data demonstrate that even a moderate dose (10 μg x 5d) of bscEGFRvIIIxCD3 was sufficient to recruit and activate lymphocytes to significantly extend survival in mice with established, late-stage EGFRvIII-expressing tumors in the brain. This result was achieved using a cumulative dose of bscEGFRvIIIxCD3 approximately 10-fold below the pharmacological equivalent of current, clinically-approved antibody therapies.

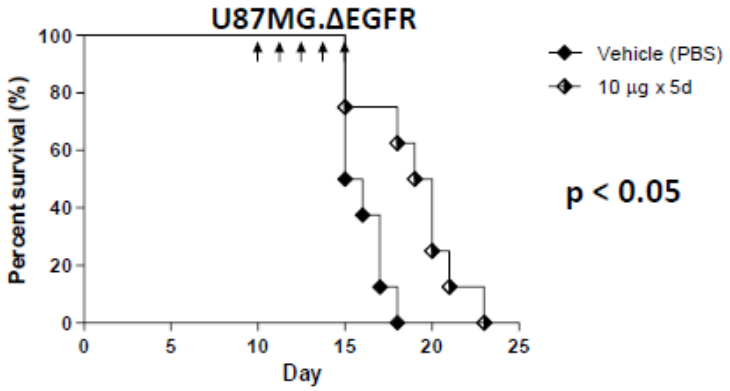


Figure 34: Delayed treatment of established intracerebral glioma with BiTE. For delayed treatment, animals were immune-reconstituted peripherally with $2x10^7$ unstimulated human PBMCs by intraperitoneal injection on day 10, followed by infusion with EGFRvIII BiTE.

Overall, these data demonstrate that systemic administration of bscEGFRvIIIxCD3 can potently activate T cells *in vivo* to achieve significant antitumor effects against EGFRvIII-expressing tumors in the CNS.

4.4 Discussion

It has been widely-demonstrated that bscAbs of the BiTE class can be used to generate potent T-cell immune responses against tumors outside the CNS. Importantly, findings in the present study demonstrated that similar responses may be achieved against established tumors in the brain. An additional advance reported here is the novel targeting of EGFRvIII with BiTE technology, making bscEGFRvIIIxCD3 the first molecule of its kind to target tumors specifically without cross-reactivity against antigens expressed normal tissues.

In general, bscAbs designed to redirect the immune system against cancer have an extensive history that has been thwarted by several shortcomings. While some alternative bscAb constructs have resulted in prohibitive toxicity due to nonspecific T-cell activation, others have been hampered by low potency, in many cases requiring extremely high E:T ratios and high concentrations of the therapeutic agent, as well as T-cell prestimulation or costimulation in order to achieve acceptable preclinical efficacy (Choi et al., 2011). By comparison, our study demonstrates that bscEGFRvIIIxCD3 has the ability to safely and specifically mediate antitumor responses that are both dose-dependent and efficacious at low E:T ratios (2.5:1), without the need for additional T-cell stimulation. Indicating its potency, a cumulative dose of just 5 μg (~0.25 mg/kg) bscEGFRvIIIxCD3 *in vivo* was sufficient to significantly prolong survival in mice with EGFRvIII-expressing tumor. Notably, this dose is roughly equivalent to 0.02 mg/kg for the average 60 kg adult (Reagan-Shaw et al., 2008); by stark contrast, recommended therapeutic doses of currently approved antitumor mAb antibodies range from 2 mg/kg (Herceptin) (Slamon et al., 2001) to up to 10 mg/kg (Avastin) (Hurwitz et al., 2004), highlighting the potential for vast improvement over currently available antibody treatments for solid tumors.

Previous studies have established that bscAbs of the BiTE class potentiate antitumor immune responses solely in the presence of cognate antigen expression by target cells (Choi et al., 2011). This is partly due to the fact that the soluble anti-CD3

scFv moiety alone is not in itself directly tumoricidal, nor does it elicit functional T-cell activation or cytokine release compared to its cross-linking mAb counterparts (Le Gall et al., 2004). As such, our *in vitro* and *in vivo* data corroborate that bscEGFRvIIIxCD3 is indeed dependent on target-cell EGFRvIII surface expression since bscEGFRvIIIxCD3 did not mediate appreciable antitumor effects in the absence of EGFRvIII expression on matched tumor cell lines. Furthermore, the need for dual-specificity was confirmed by the absence of antitumor activity both when using a control bscAbxCD3 and upon EGFRvIII-specific blockade with cognate PEPvIII peptide. Importantly, these data also support peptide blockade as a useful strategy to eliminate unintentional T-cell activation against normal tissues; given our findings, it may even be reasonable to create larger peptide constructs designed to not enter the CNS—such molecules would in theory reduce systemic toxicity, while permitting intracerebral efficacy of antibody-redirected T-cell therapies that otherwise cause toxic side-effects due to promiscuous activity against peripherally expressed antigens.

One unexpected finding in our study is the ability for systemically administered bscEGFRvIIIxCD3 to treat tumors in the CNS. To date, several attempts have been made to validate immunotherapy as a viable treatment modality for tumors in the brain; however, in the great majority of published studies, beneficial antitumor immune responses have been limited to animal models in which vaccines were administered prior to tumor challenge. By contrast, it has generally been difficult to demonstrate

successful treatment of intracerebral tumors following implantation, a fact which may partially explain the consistently poor clinical translation of immune-based interventions for patients with high-grade glioma. Such considerations should be taken into account when interpreting the results of the present study, in which survival advantages were observed in the setting of even late-stage, well-established tumors. These results provide evidence that bscEGFRvIIIxCD3 may be considerably more effective upon clinical translation than other strategies that have been tested in animal models of tumor challenge.

Due to longstanding notions of immune-privilege in the brain, previous studies of bscAbs for CNS malignancies have been limited to intralesional treatment *in vivo*, and have not been tested for efficacy by systemic administration as reported in the present study (Grosse-Hovest et al., 2005). However, a growing body of evidence supports that EGFRvIII-specific molecules may possess a special ability to accumulate in tumor tissue located in the brain, and that this phenomenon may be attributed in part to exclusive expression of the EGFRvIII mutation in malignancy. Due to their greatly reduced molecular weight, bscAbs of the BiTE class may prove even more efficient in their ability to localize intracerebrally, and studies are currently underway to further investigate this possibility. Moreover, although CNS access is certainly limited to some degree, the relative potency of the BiTE platform at extremely low doses suggests that only small

amounts may actually need to reach the tumor in order to mediate significant therapeutic effects.

We also observed in our study that therapeutic effects of bscEGFRvIIIxCD3 were associated with the accumulation of immune cells in the CNS. It has been previously shown that activated T lymphocytes have the ability to penetrate the blood-brain barrier (BBB) under even normal physiological conditions (Engelhardt and Ransohoff, 2005). While the focus of this literature has been dominated by $CD4^+$ T cells—likely due to their involvement in neuroinflammatory disease—more recent reports show that $CD8^+$ T cells also traffic into the brain and that this activity is mechanistically dependent on major histocompatibility (MHC) class I antigen presentation on cerebral endothelium (Galea et al., 2007). Interestingly, it has also been shown that EGFRvIII and other oncoproteins can be presented on the luminal surface of cerebral endothelial cells *via* microvesicular transfer (Al-Nedawi et al., 2009, Koga et al., 2005). Because bscAbs like bscEGFRvIIIxCD3 have the ability to activate T cells without the need for conventional MHC recognition, it is conceivable that interaction between T lymphocytes and luminal antigen as mediated by a bscAb may offer a unique approach to recruiting polyclonal T lymphocyte populations past the BBB. To our knowledge, the application of bscAb technology toward this end has not yet been suggested. However, similar phenomena may partially explain results from early clinical trials wherein treatment with other BiTEs led to unexplained CNS side-effects, potentially due to the peripheral activation of

T cells and subsequent ability for these cells to enter the brain (Klinger et al., 2012, Bargou et al., 2008). The implications of the discussion surrounding BiTEs, circulating lymphocytes and CNS localization are reviewed in greater detail in later chapters, and offer promising avenues for future study.

While the primary goal of this work is to propel clinical translation of a novel treatment for patients with GBM, our observations also contribute to the growing body of literature regarding T-cell activation and its importance in antitumor immunity. Further investigation will be necessary to determine whether the therapeutic benefits here are recapitulated in human studies, and whether efficacy of bscEGFRvIIIxCD3 is impacted by standard-of-care therapies for GBM including radiation and temozolomide chemotherapy, a major side-effect of which is profound lymphopenia. These areas of research may best be approached in preclinical murine models with syngeneic tumors or in novel transgenic mice possessing T cells expressing human CD3 (Kuhn et al., 2011). Our group has recently rederived this useful mouse strain, preliminary data and potential uses of which will be reviewed in upcoming chapters of this dissertation. Almost certainly, the relevance of such studies will continue to broaden as this promising new drug class progresses through ongoing clinical trials.

5 Human Regulatory T cells Kill Tumor Cells Through Granzyme-Dependent Cytotoxicity Upon Retargeting with EGFRvIII BiTE

A major mechanism by which human regulatory T cells (T_{regs}) have been shown to suppress and kill autologous immune cells is through the granzyme-perforin pathway. However, it is unknown whether T_{regs} also possess the capacity to kill tumor cells using similar mechanisms. In the last chapter, we demonstrated that bispecific antibodies (bscAbs) have emerged as a promising class of therapeutics that activate T cells against tumor antigens without the need for classical MHC-restricted TCR recognition. In the current chapter, we will show that the same bscAb targeting the tumor-specific mutation of the epidermal growth factor receptor, EGFRvIII, has the capacity to redirect human T_{regs} to kill glioblastoma (GBM) cells. This activity was found to be significantly abrogated by inhibitors of the granzyme-perforin pathway. Notably, analyses of human primary GBM also displayed diffuse infiltration of granzyme-expressing $Foxp3^+$ T cells. Together, these data suggest that despite their known suppressive functions, tumor-infiltrating T_{regs} possess potent cytotoxic mechanisms that can be co-opted for efficient tumor cell lysis.

5.1 Introduction

A major barrier that has impeded translation of efficacious immunotherapy is the inability to overcome profound immunosuppression associated with malignant disease (Zou, 2005). Regulatory T cells (T_{regs}) in particular are thought to play a central role in

tumor escape from immune-mediated rejection. One mechanism by which T_{regs} are known to suppress and even kill autologous immune cells is through the granzyme-perforin pathway (Grossman et al., 2004a). Despite this well-characterized cytotoxic capacity, whether T_{regs} can co-opt cytotoxic mechanisms to kill tumor cells has yet to be evaluated.

T-cell-activating bispecific antibodies (bscAbs)—particularly those of the bispecific T-cell engager (BiTE) subclass—represent a new therapeutic strategy that has the potential to treat even bulky, invasive disease (Bargou et al., 2008). As reviewed in detail in previous chapters, BiTEs are tandem single-chain molecules that possess dual specificity for tumor-associated surface antigens and the CD3 complex on T cells, which allows them to divalently bind and afford potent, specific target cell lysis (Choi et al., 2011). Because CD3 is universally expressed among T cells, BiTEs have the theoretical capacity to redirect and activate even T_{regs} that are elevated and present in tumors of patients with cancer.

Among the few known tumor-specific antigens, perhaps the most widely-characterized is the truncated mutant epidermal growth factor receptor variant type III (EGFRvIII). EGFRvIII is a constitutively activated tyrosine kinase that is frequently expressed on the surface of GBM and other common neoplasms but is completely absent from healthy tissues (Choi et al., 2009).

In this chapter, we demonstrate that the EGFRvIII-specific BiTE, bscEGFRvIIIxCD3, successfully redirects highly-purified T_{regs} and activates them in the presence of tumors expressing EGFRvIII. Despite their known suppressive properties, T_{regs} efficiently lysed EGFRvIII-expressing GBM *in vitro* upon redirection and activation with bscEGFRvIIIxCD3. This activity was found to be dependent on the granzyme-perforin pathway. Immunohistochemistry (IHC) analysis from human primary GBMs also displayed diffuse infiltration of activated, granzyme-producing $Foxp3^+$ cells, suggesting that T_{regs} with potent effector functions may already be present in tumors even under natural conditions.

Previous efforts to enhance antitumor immunity *via* T_{reg} depletion have been limited, in part due to an inability to efficiently eliminate suppressive cells that infiltrate tumor tissue (El Andaloussi et al., 2006). Alternatively, our data suggest that BiTEs may actually convert suppressive T_{regs} into effector cells that possess great cytotoxic potential. These findings not only highlight a new mechanism by which BiTEs may circumvent certain aspects of T_{reg}-mediated suppression, but also have broader implications with regard to the natural functional role of activated, tumor-infiltrating T_{regs} that express granzyme and perforin in the tumor microenvironment.

5.2 Materials and Methods

5.2.1 Tumor Cell Lines and Reagents

The human glioma cell line U87MG and its subline U87MG.ΔEGFR, which expresses EGFRvIII, are described elsewhere (Nishikawa et al., 1994). Characterization, production and purification of bscEGFRvIIIxCD3 as well as control antibody constructs were performed as previously described by us (Choi et al., 2013). Antibodies to CD4 (RPA-T4), CD25 (M-A251), CD69 (L78), CD152 (BNI3), Granzyme A (CB9), Granzyme B (GB11) and Perforin (δG9) were purchased from BD Biosciences. Anti-FoxP3 antibody (PCH101) and the Foxp3 Staining Buffer Set were purchased from eBioscience and intracellular staining was performed according to manufacturer instructions. Antibodies against human FoxP3 (259D, BioLegend) and granzyme B (Cat No. ab4059) were used for immunohistochemical staining.

5.2.2 T_{reg} Isolation and Preparation

All human samples were obtained at Duke University Medical Center from individuals who had given written, informed consent. Human PBMCs were prepared by density gradient centrifugation from buffy coats of healthy donor leukaphereses. Highly purified regulatory T cells were isolated from PBMCs by magnetic separation using the $CD4^{+}CD25^{+}CD127^{dim/-}$ Regulatory T Cell Isolation Kit II and autoMACS Separator (Miltenyi Biotec) according to manufacturer instructions. Following isolation, purity was confirmed by flow cytometric analysis to be between 90 to 95%

(CD25$^+$Foxp3$^+$). In certain experiments using preactivated cells, purified T_{regs} were activated and expanded with the T_{reg} Expansion Kit (Miltenyi Biotec) at a MACSiBead Particle-to-T_{reg} ratio of 4:1 according to manufacturer instructions. Cells were expanded in the presence of recombinant interleukin 2 (rIL-2) at 500 U/mL for a maximum of 14 days in the absence of rapamycin and were maintained at a purity between 90 to 95% (CD25$^+$Foxp3$^+$) (Efimova and Kelley, 2009). Following isolation and expansion, T_{regs} were verified for their ability to suppress proliferation of responder cells *in vitro* as previously described (Koristka et al., 2012).

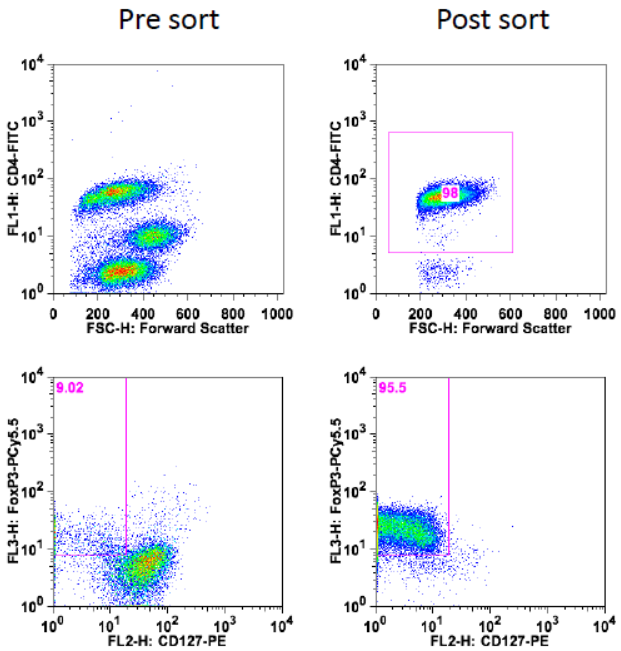


Figure 35: Magnetic bead sorting yields T_{reg} purity greater than 95% determined by phenotypic markers, FoxP3 and CD127.

5.2.3 *In Vitro* Activation and Functional Assays

Activation, proliferation, cytokine secretion and measures of specific lysis were performed as previously described by us (Choi et al., 2013). For inhibition of the granzyme-perforin axis, concanamycin A (CMA, 100 nM; Sigma), Granzyme B Inhibitor I (Z-AAD-CMK, 50 μM; Calbiochem), or ethylene glycol tetraacetic acid (EGTA, 4 mM; Calbiochem) was added to each well in a total volume of 200 μL. Blockade of FasL- and TRAIL-mediated apoptosis was carried out with antibody clones NOK-1 (25 μg/mL; BD Biosciences) and RIK-2 (25 μg/mL; BD Biosciences), respectively. Where noted, supplemental rIL-2 at 1000 U/mL was added to purified, previously-unstimulated T_{regs} during culture in order to maintain their suppressive function (Barron et al., 2010).

5.2.4 Immunohistochemistry

Tissues for immunohistochemical analysis were derived from human brain tumor biopsy material. Paraffin-embedded tissues were fixed, cut into 5 μm sections, mounted on glass slides and subjected to primary and secondary staining using the MACH 2 Double Stain 1 polymer detection kit (Biocare Medical) according to manufacturer instructions. Diaminobenzidine (DAB) and AminoEthyl Carbazole (AEC) were used to detect granzyme B and Foxp3, respectively.

5.2.5 Statistical Analysis

Groups were compared using a two-sample two-tailed t-test and statistical significance was determined at a value of $P < 0.05$.

5.3 Results

To investigate whether bscEGFRvIIIxCD3 possesses the ability to activate T_{regs}, purified $CD4^{+}CD25^{+}CD127^{dim/-}$ T_{regs} were cocultured with glioma cells expressing EGFRvIII (U87MG.ΔEGFR) and bscEGFRvIIIxCD3. T_{regs} were also cocultured with matched malignant glioma cells that do not express EGFRvIII (U87MG) to exclude the possibility that the CD3-binding portion of bscEGFRvIIIxCD3 alone was sufficient for activation. After 48 hours of coculture, T cells were harvested and examined for surface and intracellular activation markers. Expression of surface activation makers CD69 and CD25 were significantly elevated on T_{regs} stimulated by bscEGFRvIIIxCD3 specifically in the presence of U87MG.ΔEGFR. Although T_{regs} are known to constutively express elevated Foxp3 and CTLA-4, activation with bscEGFRvIIIxCD3 led to even greater expression of these markers as well, further indicating a favorable sign of activation (Miyao et al., 2012, Wing et al., 2008).

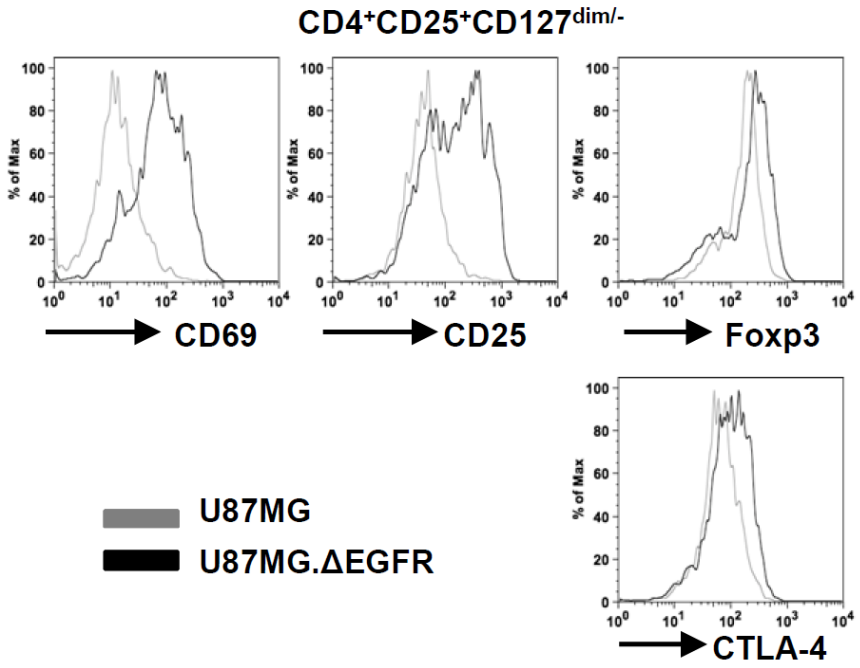


Figure 36: EGFRvIII BiTEs activate T_{regs} to upregulate surface activation makers in the presence of EGFRvIII-expressing tumor *in vitro*.
Purified $CD4^{+}CD25^{+}CD127^{dim/-}$ T_{regs} express elevated levels of activation markers CD69, CD25, Foxp3 and CTLA-4 in response to bscEGFRvIIIxCD3 specifically in the presence of EGFRvIII-expressing tumors.

Sorted T_{regs} produced greatly reduced levels of inflammatory cytokines when compared to $CD4^{+}CD25^{-}$ helper T cells in response to stimulation with bscEGFRvIIIxCD3. In addition, of the cytokines produced, redirected T_{regs} appeared to have a predominately immunosuppressive functional profile when analyzed by standard cytometric bead array analysis.

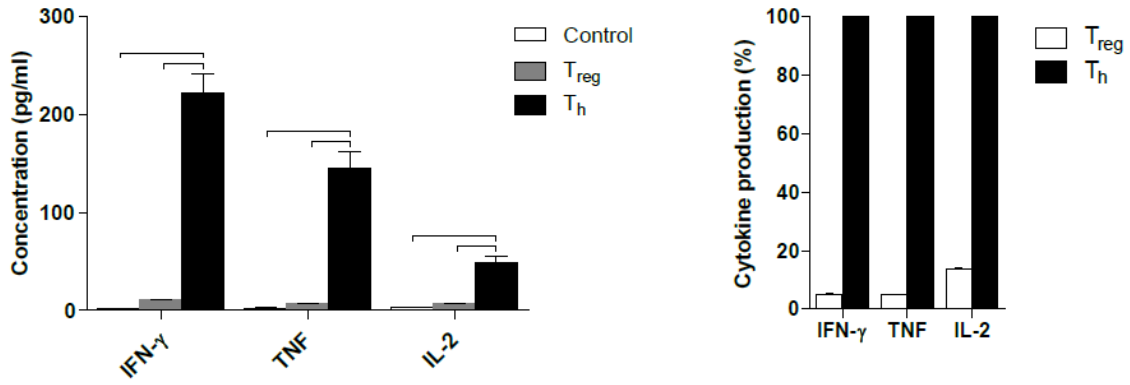


Figure 37: T_{regs} produce significantly lower levels of inflammatory cytokines compared to responder helper T cells in response to bscEGFRvIIIxCD3.
(Left panel) Supernatants from wells containing U87MG.ΔEGFR, bscEGFRvIIIxCD3 and $CD4^+CD25^+127^{dim/-}$ T_{regs} contained significantly lower levels inflammatory cytokines compared to wells in which responder cells consisted of purified $CD4^+CD25^-$ helper T cells (T_h). Control bars represent cytokine secretion of T_{regs} in the presence of non-specific BiTE and U87MG.ΔEGFR. All tests were performed in triplicate wells and independently repeated. Horizontal bars represent a statistical significance of $P < 0.05$. (Right panel) This same data is presented normalized with respect to cytokines secreted by T_h cells.

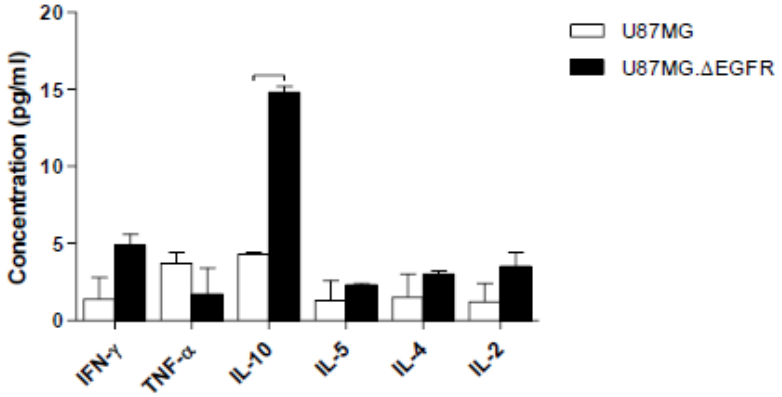


Figure 38: Cytokine profile of redirected T_{regs} demonstrates secretion of IL-10 in the presence of EGFRvIII BiTE and target cells expressing EGFRvIII.

Moreover, T_{regs} also remained refractory to proliferative signals in response to stimulation under these conditions, indicating that activation *via* bscEGFRvIIIxCD3 appears to have similar effects on T_{reg} activity that would be generally expected with standard methods of T-cell stimulation *in vitro.*

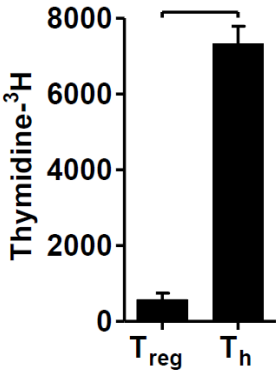


Figure 39: T_{regs} remain refractory to proliferative signals in response to simulation through EGFRvIII BiTE.
Proliferation of Tregs and Th in response to bscEGFRvIIIxCD3 and solid phase EGFRvIII as measured by 3H-thymidine incorporation demonstrates that proliferative defects in the Treg compartment persist following activation with bscEGFRvIIIxCD3. All tests were performed in triplicate wells and independently repeated. Horizontal bars represent a statistical significance of $P < 0.05$

As a class of therapeutics, bscAbs are known to bypass a number of tumor-associated mechanisms of immune escape by allowing effector T cells (T_{effs}) to detect and eliminate tumor cells without the need for conventional antigen-presentation and MHC-peptide recognition. Based on recent findings that increasing the strength of cognate antigen stimulation may actually allow T_{effs} to overcome the suppressive effects of T_{regs} on cytotoxicity (Gobel et al., 2012), we sought to determine whether the EGFRvIII-

specific bscAb would similarly redirect T_{effs} against target tumor cells despite the presence of suppressive T_{regs} *in vitro.* In a standard ^{51}Cr release assay using freshly thawed, unstimulated human PBMC as effector cells, incubation with bscEGFRvIIIxCD3 led to the efficient lysis of EGFRvIII-expressing tumor cells as previously described by us. Interestingly, we found that the addition of T_{regs} (effector:T_{reg}, 1:1) did not negatively impact redirected cytotoxicity but instead significantly enhanced overall specific lysis against EGFRvIII-expressing tumor cells *in vitro*.

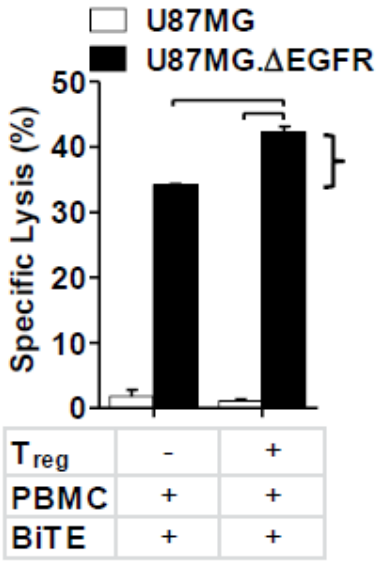


Figure 40: T_{regs} do not suppress effector cell lysis against EGFRvIII-expressing glioma upon redirection with EGFRvIII BiTE.
Standard ^{51}Cr-release demonstrates specific lysis of U87MG.ΔEGFR over U87MG by unstimulated human PBMCs and BiTE that is significantly elevated in the presence of T_{regs} at an effector to T_{reg} ratio of 1:1. All tests were performed in triplicate wells and independently repeated. Horizontal bars represent a statistical significance of $P < 0.05$.

Although a number of mechanisms could account for this phenomenon, certainly one possible explanation is that T_{regs} may actually be acting as cytotoxic effectors against tumor cells in the presence of bscEGFRvIIIxCD3. To formally test this, we sought to determine whether highly purfied T_{regs} alone could mediate measureable specific lysis against tumor cells. Indeed, when using purified, previously unactivated T_{regs} as effector cells *in vitro*, we found that addition of EGFRvIII-specific BiTE yielded significant antitumor effects against target cells expressing EGFRvIII. Interestingly, the magnitude of cytotoxicty observed by purified T_{regs} alone approximated the additional cytotoxicity that had been observed upon addition of T_{regs}, notated by brackets in the respective figures

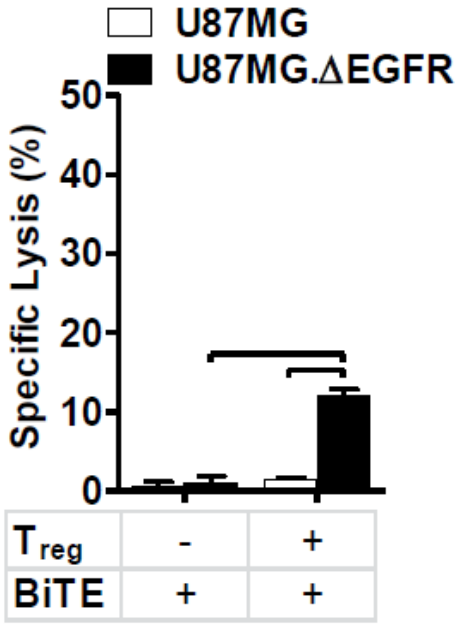


Figure 41: T_{regs} acquire the ability to directly kill tumor cells upon redirection with the EGFRvIII BiTE.

When purified, $CD4^{+}CD25^{+}CD127^{dim/-}$ T_{regs} alone were also observed to lyse target EGFRvIII-expressing tumor cells upon redirection with BiTE (18 hours; E:T ratio 20:1; [BiTE] 10 μg/mL; rIL-2). Vertical bracket illustrates comparable magnitudes of cytotoxicty observed by purified T_{regs} alone with additional cytotoxicity that had been observed upon addition of T_{regs}. All tests were performed in triplicate wells and independently repeated. Horizontal bars represent a statistical significance of $P < 0.05$.

Upon activation, T_{regs} are known to possess sigificant suppressive capacity, at least in part due to their ability to efficiently induce high levels of granzyme B expression (Efimova and Kelley, 2009). The perforin-granzyme pathway is a well-characterized mechanism by which T_{regs} are known to actually kill effector cells, thereby inhibiting effective immune responses (Grossman et al., 2004a, Grossman et al., 2004b). However, because the EGFRvIII-specific BiTE has the theoretical potential to activate

and redirect all cells expressing CD3—including even suppressive T_{regs}—we hypothesized that this molecule might also coopt T_{regs} to kill EGFRvIII-expressing tumor cells, specifically by redirecting their perforin-granzyme-mediated mechanisms of immune suppression to instead destroy target cancer cells.

To test this hypothesis, we first designed experiments to determine the impact of bscEGFRvIIIxCD3 on the expression of perforin and granzymes in T_{regs}. PBMCs were gated on $CD4^+CD25^+Foxp3^+$ cells prior to flow cytometric analysis for intracellular perforin and granzyme expression. While unactivated T_{regs} expressed only low levels of perforin, granzyme A (GrA) and granzyme B (GrB), T_{regs} activated in the presence of bscEGFRvIIIxCD3 and EGFRvIII-expressing target cells exhibited marked upregulation of the perforin-granzyme pathway.

Importantly, consistent with what has been shown for in the setting of activation, T_{regs} continued to supress responder cell proliferation *in vitro*, indictating retention of their typical properties following engagement with the EGFRvIII BiTE.

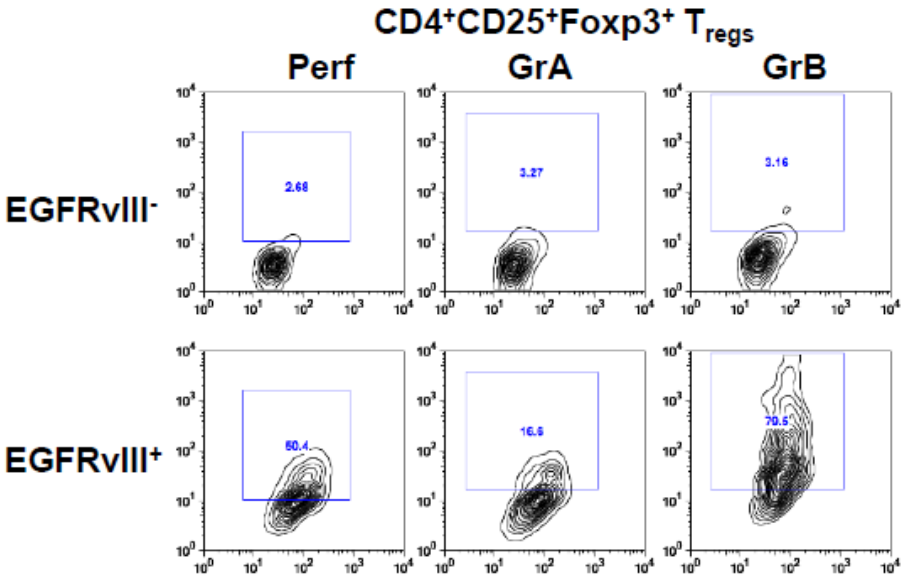


Figure 42: T_{regs} activated by bscEGFRvIIIxCD3 exhibit marked upregulation of the perforin-granzyme pathway.

Human PBMCs were incubated in the presence of U87MG ($vIII^-$, top) or U87MG.ΔEGFR ($vIII^+$, bottom) and bscEGFRvIIIxCD3, harvested and stained for flow cytometric analysis. $CD4^+$ cells were isolated and gated for high CD25 and Foxp3 expression and then analyzed for Perf, GrA and GrB expression with positivity determined by isotype control. Plots are representative of at least three repeated experiments.

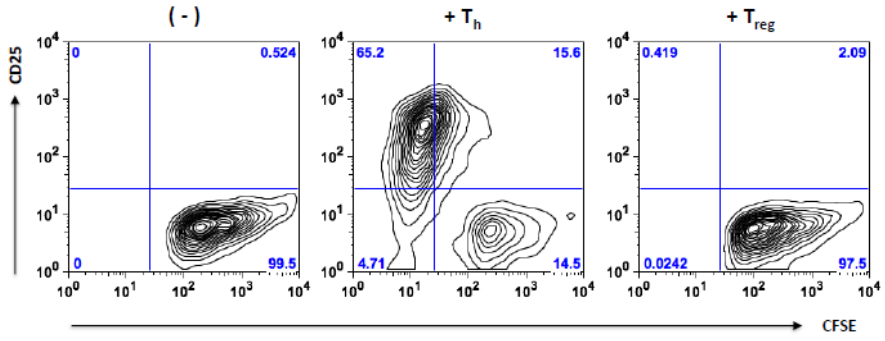


Figure 43: Redireted T_{regs} suppress responder cell proliferation followed by TCR engagement by bscEGFRvIIIxCD3.

Given that even previously unactivated T_{regs} yielded antitumor effects *in vitro* with presumeably lower levels of perforin and granzyme expression, we sought to determine whether preactivated T_{regs}—which are actually thought to more closely resemble highly suppressive T_{regs} associated with tumors (Gobert et al., 2009)—also possessed the capacity to kill tumor cells. Indeed, our data demonstrate that preactivated T_{regs} efficiently lyse target cells expressing EGFRvIII when redirected with EGFRvIII-specific BiTE. Importantly, this effect was not detected in the presence of a nonspecific control bscAb or T_{regs} alone, further demonstrating the specifity of this cytolytic activity.

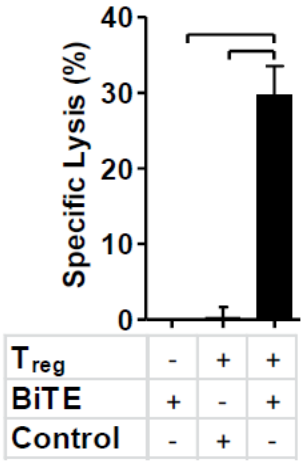
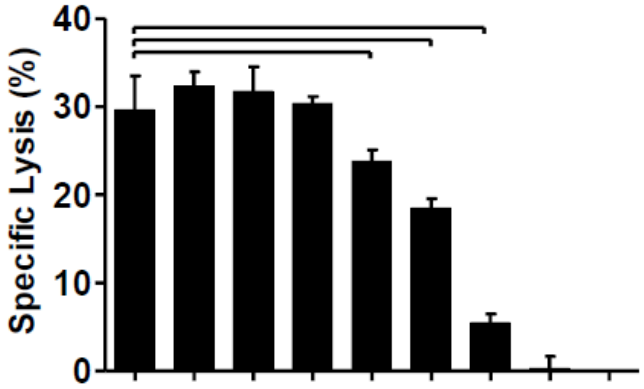


Figure 44: Activated T_{regs} possess enhanced cytotoxic activity against EGFRvIII-expressing tumor in the presence of EGFRvIII BiTE.
Upon redirection with bscEGFRvIIIxCD3, but not a non-specific control bscAb (Control), activated T_{regs} demonstrate enhanced lysis against EGFRvIII-expressing tumor (T_{reg}:target, 20:1; incubation time 18 hours; [BiTE] 10 μg/mL). All tests were performed in triplicate wells and independently repeated. Horizontal bars represent a statistical significance of $P < 0.05$.

Moreover, redirected T_{reg}-mediated lysis was shown to be actually dependent on the perforin-granzyme pathway, since cytotoxicity was not impacted by TRAIL or FasL blockade, but significantly abrogated in the presence of previously-characterized inhibitors of perforin- and granzyme-mediated cytotoxicity.



T_{reg}	+	+	+	+	+	+	+	+	-
BiTE	+	+	+	+	+	+	+	-	+
RIK-2	-	+	-	-	-	-	-	-	-
NOK-1	-	-	+	-	-	-	-	-	-
IgG1	-	-	-	+	-	-	-	-	-
Z-AAD-CMK	-	-	-	-	+	-	-	-	-
EGTA	-	-	-	-	-	+	-	-	-
CMA	-	-	-	-	-	-	+	-	-

Figure 45:: T_{reg}-mediated antitumor effects are dependent on the granzyme-perforin pathway.

Specific lysis against target tumor cells expressing EGFRvIII is not significantly inhibited by blockade of Fas ligand- and TRAIL-mediated apoptosis but is significantly abrogated by partial inhibitors of the granzyme-perforin pathway, Z-AAD-CMK, EGTA and CMA. Pairwise comparisons with respect to T_{reg}, BiTE and inhibitors of the granzyme-perforin pathway were made. All tests were performed in triplicate wells and independently repeated. Horizontal bars represent a statistical significance of $P < 0.05$.

Adding clinical relevance to these findings, infiltrating Foxp3^{+} T_{regs} in human glioma samples were actually found to coexpress detectable levels of granzyme B by immunohistochemical analysis (**Figure 4C**), suggesting that activated T_{regs} with potent effector functions may be present within GBMs, and could potentially serve as effector cells upon infusion with T-cell-engaging bscAb therapies *in vivo*.

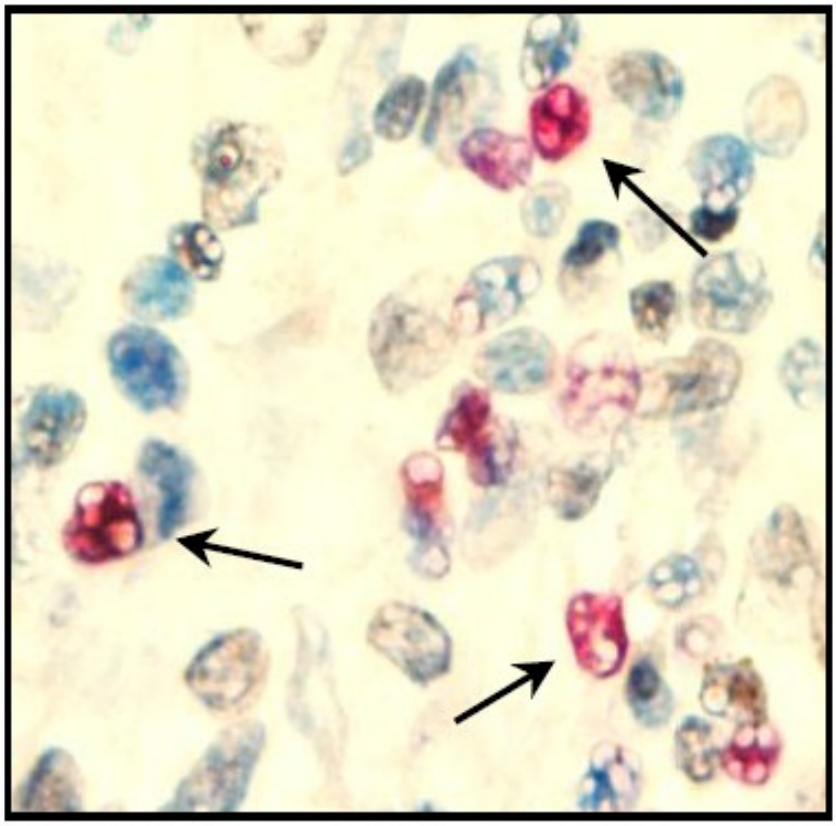


Figure 46: IHC analysis of human GBM for T_{regs} and granzyme B.
IHC analysis of human GBM shows diffuse infiltration of Foxp3^{+} T_{regs} (AEC) expressing detectable levels of granzyme B (DAB).

5.4 Discussion

In light of recent findings demonstrating that T_{regs} may actually be required for the priming of high-avidity $CD8^+$ T-cell responses (Pace et al., 2012), innovative methods to reappropriate the T_{reg} compartment without ablating these cells completely could provide an attractive alternative to currently available depletive strategies. As such, we have provided for the first time evidence and a mechanism by which T_{regs} might be redirected to kill tumor cells through engagement with a bscAb. One previous study has explored the impact of bscAbs on $T_{regs,}$ and demonstrated that bscAb-mediated activation of T_{regs} suppresses effector cell proliferation and abrogates antitumor efficacy (Koristka et al., 2012). However, the direct effects of bscAb-redirected T_{regs} on target tumor cells were not discussed. Furthermore, because their work employed the use of rapamycin to expand T_{regs} *ex vivo*—an additive which is known to completely suppress GrB expression in T_{regs} (Efimova and Kelley, 2009)—it may ultimately not be feasible to directly compare the results of our studies. In the current study, redirected cytotoxicity was dependent on the presence of bscEGFRvIIIxCD3; though, it may be reasonable to suspect that similar results might be observed upon even endogenous TCR-specific engagement of granzyme-producing T_{regs} present in the tumor microenvironment. Importantly, this work opens a new discussion regarding such potential mechanisms, which will need to be further explored across broader areas of autoimmunity and immunotherapy where T_{reg} biology is relevant.

6 Human CD3ε Transgenic Mice Provide a Novel System for Evaluation of Bispecific Antibodies

6.1 Introduction

As detailed in previous chapters, to test bscEGFRvIIIxCD3 *in vivo* we chose an NSG mouse model, which has the distinct advantage of evaluating drug candidates in an animal system using human tumor tissue, with the potential to directly translate the therapeutic molecule of interest into clinical studies. Indeed, initial preclinical studies of the first-in-class CD19-targeted BiTE now in clinical trials were originally performed in immunocompromised mouse models, the main limitations of which have been described elsewhere (Dreier et al., 2003). Briefly, one of the major drawbacks of this approach includes the need to supply human effector T cells, which is further compounded by the fact that human cells often have a limited functional half-life in the murine background. However, taking into account these considerations, it may be reasonable to conclude that our results in fact underestimate the full potential of bscEGFRvIIIxCD3, especially given that—in a realistic clinical scenario—circulating polyclonal T cell populations should be present in great number and persistent throughout the body.

Regardless, although early results against xenografts certainly provide evidence of efficacy, the species-restricted activity of our BiTE—which is reactive only with human lymphocytes—not only impedes proper optimization for safety and efficacy, but also limits the pursuit of numerous mechanistic studies that would only be

appropriately examined in hosts with endogenous immunity. Importantly, other T-cell activating antibodies have historically met with unforeseen toxicity when translated in early clinical trials, at least in part due to the lack of appropriate immunocompetent rodent models possessing surface molecules of equivalent binding affinities and function to those found in humans (Attarwala, 2010).

Thus, the challenge of evaluating human-specific agonist antibodies in animal models has represented a major bottleneck in the translation of immune-based therapies. To address this issue, transgenic mice have recently been engineered to express human CD3 on the surface of T lymphocytes, thus providing a much-needed platform for preclinical evaluation of therapeutics (Kuhn et al., 2011). It has been previously suggested that these mice would provide the ideal system for preclinical evaluation of BiTEs (Thompson et al., 2009), though the advantages of using this animal model have not yet been realized to date. When heterozygous, CD3 transgenic mice are reported to be immunocompetent and possess fully functional peripheral T cells that respond to stimulation through both human and murine CD3 (Wang et al., 1998, Weetall et al., 2002). Given the promise of our EGFRvIII targeted BiTE molecule in xenogeneic models, we decided to re-derive this valuable mouse colony to perform studies that would support relevant preclinical development. The current chapter will report preliminary data obtained to date in this model, and suggest potential avenues of study that may be pursued using this preclinical system.

6.2 Materials and Methods

6.2.1 Mice and Tumor Cell Lines

C57BL/6J were obtained from Jackson Laboratories. CD3 transgenic mice (tgε300) were transferred to Duke Division of Laboratory Animal Resources as embryos as a kind gift from Dr. Cox Terhorst (Beth Israel Deaconess Medical Center) and implanted into surrogate mothers for rederivation. Mice were maintained and bred under pathogen-free conditions at Duke University Medical Center (DUMC). All animal experiments were performed according to protocols approved by the Duke University Institutional Animal Care and Use Committee (IACUC).

6.2.2 Temozolomide Preparation

TMZ (Temodar, Schering-Plough and Best Pharmatech) was dissolved in a solution of 85% saline and 15% dimethyl sulfoxide (DMSO). Mice were weighed and injected intraperitoneally with a calculated dose daily for 5 days as indicated.

6.2.3 Peripheral Blood Draws and Complete Blood Counts

Retro-orbital eye bleeding was used to collect 50-100 μl blood into heparinized tubes for complete blood count (CBC) and flow cytometric analyses. CBCs were performed by the Duke Veterinary Diagnostic Laboratory on a VetScan HM5 Hematology Analyzer (Abaxis Inc).

6.2.4 Flow Cytometric Analysis

Antibodies to CD3 (145-2C11), CD4 (L3T4), CD8 (53-6.7), and CD16/32 (2.4G2), as well as appropriate isotype controls, were obtained from BD Pharmingen (San Diego, CA). Anti-Foxp3 (FJK-16s) was obtained from eBioscience (San Diego, CA). Whole blood obtained by retro-orbital bleeding was analyzed for T cells, $CD8^+$ T cells and $CD4^+$ T cells as follows: samples were incubated with a cocktail of antibodies in 150 μL FACS buffer in the dark for 15 minutes at room temperature. Red blood cells (RBCs) were lysed and cells were fixed using 1 ml 1X BD FACS Lysing Solution (BD Bioscience, Cat# 349202) and incubated overnight at 4 ºC. Cells were then washed and re-suspended in 2% paraformaldehyde and submitted to flow cytometry analysis.

6.3 Results

6.3.1 Characterizing the Human CD3ε Transgenic Mouse Model

We first sought out to determine whether splenocytes from our CD3ε transgenic mice (C57BL/6J background) expressed human CD3 following the rederivation procedure. These mice were previously reported to express both mouse and human CD3ε on the surface of circulating T cells, and indeed, we found this to be the case. Whereas splenocytes from wild-type C57BL/6J demonstrated only murine CD3ε expression when co-stained for both human and murine CD3ε by FACS, almost all splenocytes from transgenic mice that were positive for murine CD3ε were shown to express surface human CD3ε as well.

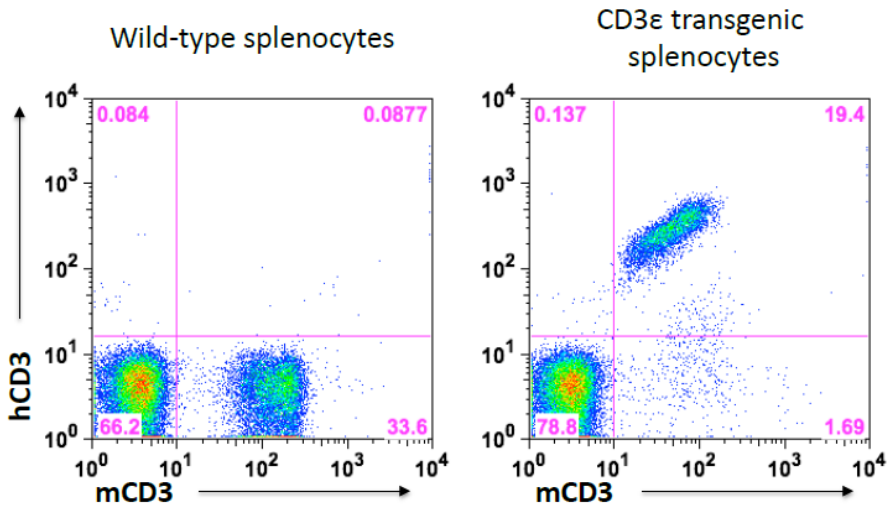


Figure 47: Analysis of surface CD3ε expression in splenocytes from human CD3ε transgenic mice.
Splenic single cell suspensions were examined by FACS analysis for both human and murine CD3ε using antibody clones OKT3 and 145-2C11, respectively.

Following our detection of human CD3ε surface expression in splenocytes of CD3ε transgenic mice, we performed experiments to determine the functional properties of the human CD3ε transgene gene product in murine T cells *in vitro*. Towards this end, we isolated splenocytes and tested several metrics of function in response to activating signals through solid phase anti-human or anti-mouse CD3-specific agonistic antibodies, OKT3 and 145-2C11, respectively. In accord with previously published data regarding this mouse strain, we found that our rederived, transgenic mice possessed T cells that were capable of functional responses upon appropriate stimulus. Importantly, while wild-type splenocytes were solely responsive to CD3ε engagement by 145-2C11, splenocytes from human CD3ε transgenic mice responded to stimulation with both 145-

2C11 and OKT3, as measured by assays of proliferation as well as secretion of inflammatory cytokines TNF-α and IFN-γ.

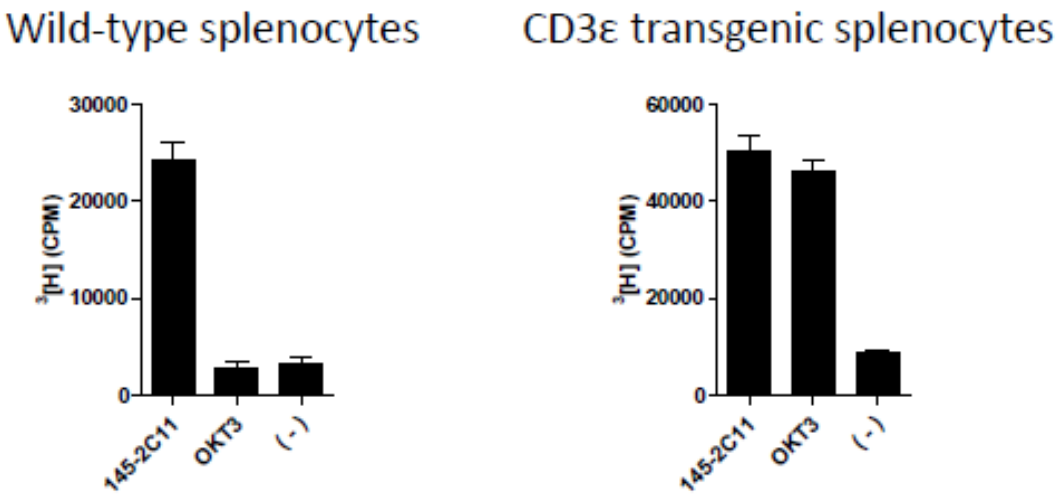


Figure 48: Proliferative responses of wild-type and CD3ε transgenic splenocytes activated by solid phase anti-CD3 agonistic antibodies.

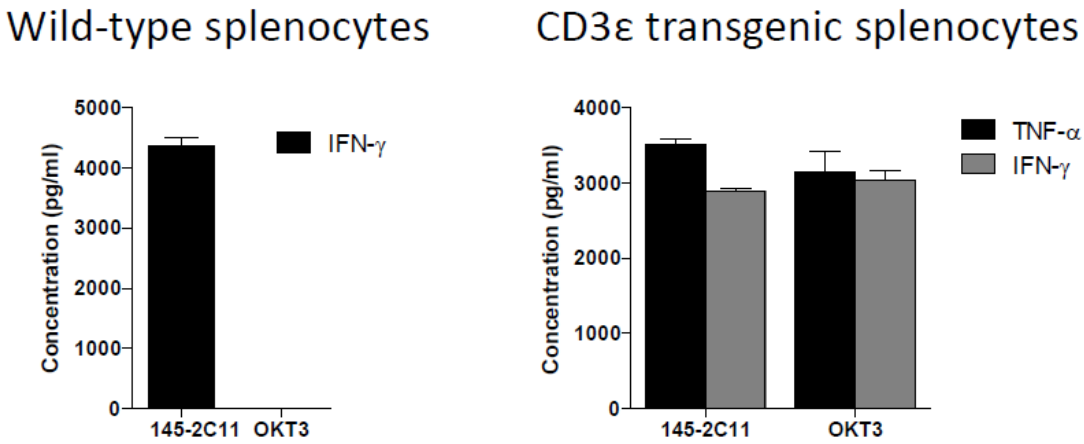


Figure 49: CBA analysis of supernatants from wild-type and CD3ε transgenic splenocytes activated by solid phase anti-CD3 agonistic antibodies.

6.3.2 Ongoing Studies to Evaluate BiTEs in the Context of Lymphodepletive Chemotherapy

One factor that may play a significant role in the ability of BiTEs to successfully treat brain tumors is the availability of circulating effector T cells. This is of particular concern in patients with glioma, since a prominent side effect of current standard-of-care temozolomide chemotherapy is the induction of profound lymphopenia. Given that BiTE-mediated antitumor efficacy is known to rely on the redirection of T cells, it is reasonable to speculate that depletion of this cellular compartment may compromise the efficacy of the BiTE therapeutic platform. This line of reasoning is a classic illustration of how immunocompetent preclinical models—such as the CD3ε transgenic system—are essential to appropriately evaluate immune-modulatory therapies, given that xenograft systems cannot accurately reflect the physiological milieu of an immune replete host, especially during recovery from lymphodepletive chemotherapy.

In our group, we have recently developed murine doses of temozolomide that are designed to recapitulate clinically relevant chemotherapy-induced lymphodepletion in the C57BL/6J mouse model. Our data demonstrated that treatment with a non-myeloablative temozolomide dose (60 mg/kg x 5 days) leads to a significant T-cell lymphopenia in mice. Flow cytometric analysis of peripheral blood revealed that both $CD4^+$ and $CD8^+$ were profoundly depleted immediately after cessation of temozolomide administration, and that these cells either remained depressed or experienced protracted recovery over the course of 30 days.

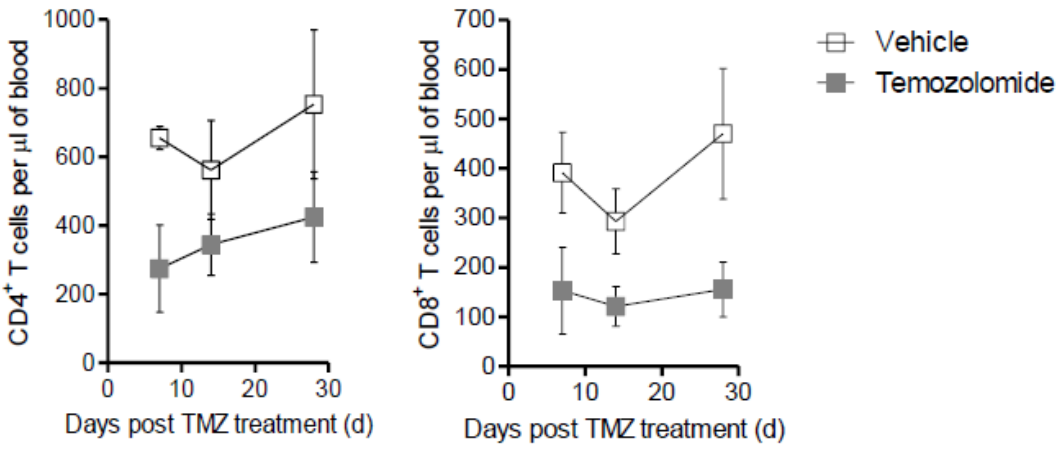


Figure 50: Temozolomide chemotherapy results in depletion of circulating T cells. To evaluate the impact of temozolomide chemotherapy on circulating T cells, C57BL/6 mice (n = 5) were treated with vehicle or temozolmide (60 mg/kg x 5 days). Mice were bled retro-orbitally on days 7, 14 and 28 after termination of TMZ administration and submitted to flow cytometry analysis. Absolute numbers per µL of blood were calculated using Flowcount beads (Beckman Coulter).

6.4 Discussion

Although we have not yet formally evaluated the efficacy of BiTEs in the context of an immune competent host, there are several potential advantages to developing an murine model that could eventually be applied towards this end. For example, in addition to the fact that lymphodepletion may antagonize BiTE-mediated antitumor responses *in vivo*, emerging hypotheses also posit that peripheral depletion of otherwise abundant $CD3^+$ cells may actually promote the intracerebral accumulation of BiTEs at tumor sites in the brain. This theory is based on the idea that a vast population of circulating CD3-expressing cells may otherwise sequester BiTE molecules in the

periphery, hampering their ability to localize to tumors in the brain at therapeutically relevant quantities. Since BiTEs have been shown to mediate antitumor efficacy even at exceedingly low effector-to-target ratios, lymphodepletion may provide a useful strategy to promote intracerebral localization of BiTEs while maintaining an acceptable level of effector T-cell activity *in vivo*.

Overall, we believe that experiments performed in the syngeneic system will at least corroborate our results obtained in the xenograft model, and have already mentioned reasons why it may actually be superior in terms of achieving therapeutic effects. Conversely, however, we may ultimately find that our immunocompetent model may yield less dramatic results, in which case the CD3ε transgenic system could still be leveraged to explore variables that may improve our strategy for translation and clinical trial design. For instance, whereas EGFRvIII BiTE localizes rapidly and efficiently to intracerebral xenograft tissue, this level of accumulation may not be achieved when tumor and stroma cells grow together in the syngeneic setting. If this occurs, we could explore the opportunity to enhance intracerebral localization of both circulating T cells and BiTE—a subject that is discussed in greater detail in the next chapter of this dissertation—or attempt local injection through existing preclinical models of convection-enhanced delivery. In addition, we can also further our understanding of the potential impact of immunosuppressive factors (*e.g.*, T_{regs}, myeloid-

derived suppressor cells, cytokines) on BiTE-mediated activity, which can only be evaluated with any fidelity in the syngeneic, immunocompetent setting.

7 Early Clinical Translation of EGFRvIII BiTEs for Patients with Malignant Glioma

As introduced briefly in previous chapters, in order for BiTEs to achieve their full potential in patients, both BiTEs and effector T cells will need to have access not only to areas of permeable, bulky tumor, but also to invasive areas of infiltrative tumor thought to reside beyond an intact BBB. Importantly, substantial rationale exists to support that both BiTEs and BiTE-activated T cells may actually possess the unique ability to penetrate the BBB. Fortunately, experiments designed to explore these phenomena are ideally suited to emerging phase 0/1 microdosing clinical trials, which enable pharmacokinetic assessment of novel therapeutics in humans, without the need to submit a Investigational New Drug (IND) application.

7.1 Mechanisms by which BiTEs May Penetrate the Blood-Brain Barrier

A novel therapeutic concept in this chapter is the hypothesis that BiTEs have the capacity to accumulate beyond the BBB by two potential mechanisms: (1) BiTEs may "hitchhike" on activated T cells that are known to frequently enter and circulate through the CNS and (2) BiTE may accumulate in IC tumors due to the presence of a cognate "antigen-sink."

While circulating naïve T cells do not typically penetrate the CNS, activated T cells are known to traffic frequently past the BBB and perform routine immune surveillance in the CNS (Hickey et al., 1991). Furthermore, it has previously been shown

that particles bound to the surface of antigen-specific T cells have the ability to localize to tumors and accumulate there (Cole et al., 2005). Whether this mechanism could be used to enhance localization of a macromolecule like a BiTE has not been studied to date, but has potentially far-reaching consequences in the fields of BBB biology and therapeutic delivery. Notably, it has also been shown that upon even natural stimulation in the periphery, T cells gain the ability to track into the CNS (Odoardi et al., 2012). Similarly, BiTEs may also play an important role in the localization and retention of effector T cells at IC sites. Recent data have shown that BiTEs targeted against systemically-expressed antigens like CD19 lead to peripheral activation of effector-memory T cells (Klinger et al., 2012, Bargou et al., 2008), which was temporally associated with subsequent unexplained, but transient, CNS side effects in multiple patients. Consistent with this, our data demonstrate that in preclinical models, intravenous treatment with the EGFRvIII-specific BiTE results in the accumulation and diffuse infiltration of lymphocytes to intracerebral EGFRvIII-expressing tumors. In contrast, this phenomenon was completely absent in mice receiving a phosphate-buffered saline (PBS) vehicle control, suggesting that trafficking of T cells to the target EGFRvIII antigen was at least in part dependent on presence of the BiTE. T cells thought to cause CNS symptoms in patients receiving the CD19 BiTE consisted largely of activated, effector-memory T cells, which are also known to be precisely the T-cell phenotype that is critical in an effective antitumor response; although such infiltrating T

cells did not naturally possess specificity to attack cells expressing tumor antigens in the CNS, an EGFRvIII BiTE would in theory have the ability of redirecting these highly-activated T cells—that otherwise possess agnostic T-cell receptor (TCR) restriction—against tumors in the brain.

Another novel hypothesis that may play a role in the ability of BiTEs to accumulate past the BBB is dependent on the presence of an intracerebral antigen sink. This concept refers to the ability of an antibody to accumulate over time in the proximity of its cognate antigen, if and only if this cognate antigen is not also expressed in other tissues outside the CNS. As described briefly in Chapter 1 , although access to the CNS is certainly limited to some extent, it has been known for some time that tiny amounts of peripherally circulating antibodies can in fact be detected in the CNS—between 0.1% to 1% of that found in serum (Freund, 1930). Thus in the absence of cross-reactivity with systemic antigens, it is thought that small amounts of highly-specific BiTE treatments may penetrate the CNS through a passive mechanism of diffusion, and be retained there over time to reach therapeutically relevant quantities. This theory of a functional IC antigen sink is corroborated by studies in which infusions with radiolabeled monoclonal antibody specific for EGFRvIII demonstrated tumor-specific uptake and high levels of BBB penetration in patients with GBM (Scott et al., 2007a). In addition, recent data has been published recent clinical trials, in which almost all patients who possessed potent humoral responses to an EGFRvIII peptide vaccine no longer expressed the EGFRvIII

tumor antigen in their tumors at recurrence (Sampson et al., 2010, Sampson et al., 2011). These data, along with the fact that BiTEs appear effective against invasive intracerebral tumors (Choi et al., 2013) certainly suggest that a tumor-specific humoral response can mediate therapeutic effects at an intracerebral tumor site. Importantly, the CD3 antigen present on circulating T cells should not provide a significant source of peripheral antigen in patients with GBM, given the profound lymphodepletion associated with standard-of-care temozolomide chemotherapy; however, formal evaluation of this potential phenomenon could be pursued, particularly in representative preclinical models such as the novel CD3ε transgenic system detailed in the previous chapter.

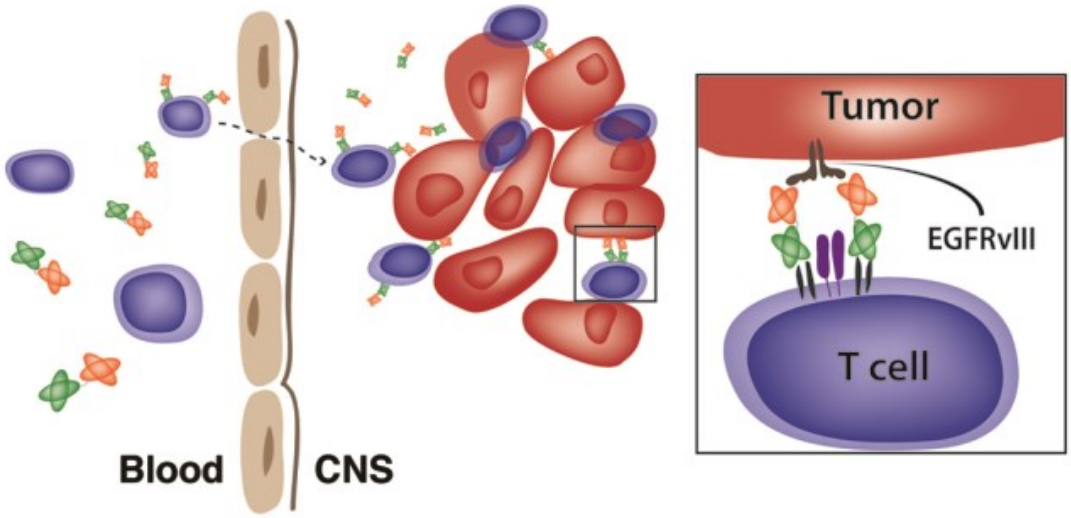


Figure 51: Mechanisms by which BiTEs may localize to intracerebral tumors. The systemic administration of an EGFRvIII BiTE results in its successful localization to EGFRvIII-expressing brain tumors and in the activation of T cells to sustain potent antitumor immune responses. The EGFRvIII-targeting BiTE may gain access to the central nervous system on the surface of activated T cells or may accumulate independently, owing to specific interactions with the cognate EGFRvIII antigen on brain tumor cells.

7.1.1 Cre-Inducible Transgenic Murine Models for the Study of Intracerebral Localization

Although our previous experience supported that peripheral administration with EGFRvIII BiTE was sufficient to treat orthotopic glioma xenografts *in vivo,* we sought to validate that intracerebral localization would be maintained under even more stringent conditions. Thus, we attempted to visualize BiTE molecules as they localized to an especially invasive, cancer stem-like GBM model that we feel closely recapitulates the BBB as it exists natural in the tumor microenvironment. Using a cranial window model

followed by intravital *in vivo* confocal microscopy, we observed that intravenously administered, fluorochrome-labeled BiTE accumulated in EGFRvIII expressing intracranial tumors, but not in adjacent normal brain from the same animal. Importantly, appreciable localization was only observed over a 24 hour period, rather than in the time immediately following injection, implying that penetration of the BBB was limited in our model compared to what would be expected for peripheral tumors or "leaky" xenograft models that have been published elsewhere. Based on these observations, there seems to be mounting support that EGFRvIII-targeted BiTEs have the capacity to successfully accumulate past the BBB to an intracerebral antigen sink.

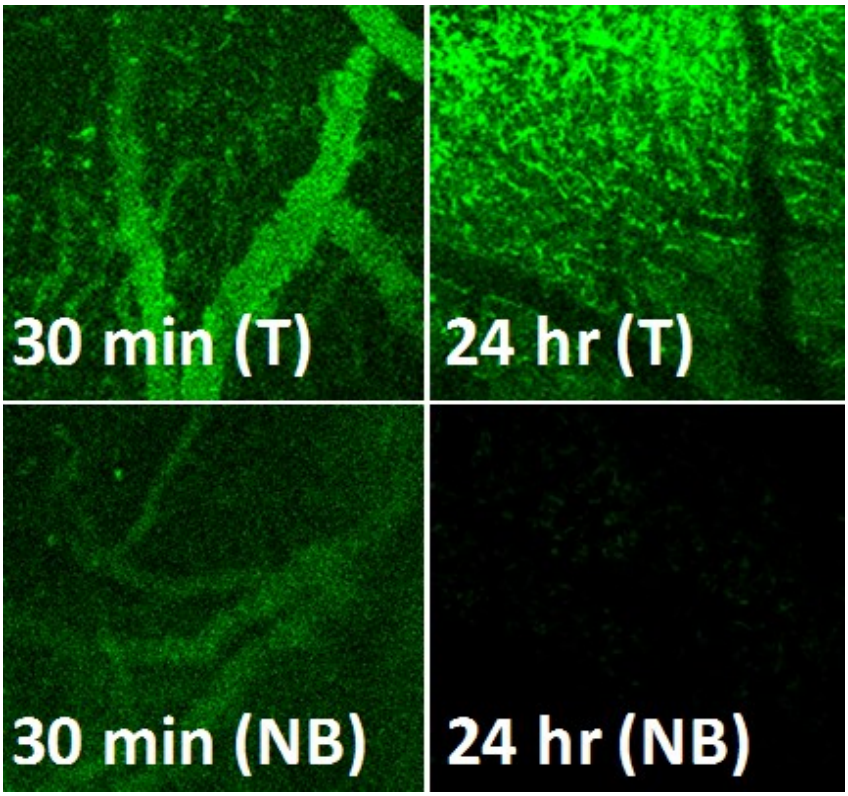


Figure 52: Intracerebral localization of BiTE to EGFRvIII-expressing glioma through cranial window.
Following engraftment of EGFRvIII-expressing GBM, surgically implanted glass windows were used for chronic intravital microscopy. After 10 days, mice were treated with intravenous EGFRvIIIxCD3 BiTE conjugated to Alexa-488. *In vivo* images were then obtained with a fluorescent confocal microscope at 30 minutes and 24 hours in either areas of tumor (T) or normal brain (NB).

As a brief aside, a number of preclinical models have also been developed that incidentally support the theoretical CNS antigen-sink hypothesis. Perhaps most convincing are experiments in which diphtheria toxin (DT) was administered to rodents with Cre-inducible diphtheria toxin receptor (DTR) expression solely in oligodendrocytes (Buch et al., 2005). Intraperitoneal DT injections in these mice led to

efficient intracerebral localization and myelin loss in the CNS. Importantly, while DT (~60 kDa) is known to cross the BBB of humans through receptor-mediated endocytosis, this protein is conversely—restricted from the brains of rodents due to species specific restriction (Wrobel et al., 1990). Because DTR is not naturally expressed murine tissues, the selective localization and ablation of oligodendrocytes in this model provides evidence that antigen-sinks may allow for accumulation of even larger protein molecules beyond the BBB.

In order to formally explore mechanisms by which T cells or BiTEs may penetrate a fully intact BBB, this well-characterized Cre-inducible transgenic mouse model can also be used to drive expression of EGFRvIII in various murine tissues of interest. The advantage of this model is ideal given that it permits anatomically-limited expression of an antigen while excluding any potential disruption of the BBB due to the presence of a tumor mass. Although this type of mouse model has been previously described (Zhu et al., 2009), the use of this system to study antigen-specific localization of T cells and or antibody-based macromolecules would be novel and innovative. These areas of future study, as well as those that may be performed in conjunction with human CD3ε transgenic mice, may yield useful preclinical data and mechanistic principle to inform future approaches to drug delivery and clinical trial design.

7.2 Phase 0/1 Microdosing Study of EGFRvIII BiTEs in Patients with Recurrent EGFRvIII^{+} Glioblastoma

Microdosing is a recent mechanism endorsed by the FDA for the quicker access of new medicine and reduced attrition of translation at the later stages of preclinical drug development. The implementation of these studies was based on the need to address the discordance between major scientific advances and limited drug development. The concept of microdosing involves the use of extremely low doses of a drug to define the pharmacokinetic profile of the medication in human subjects, and this strategy is currently used by 15 of the 20 largest pharmaceutical companies in drug development (Tewari and Mukherjee, 2010). Importantly, despite its promise, microdosing has been underutilized at academic centers to date.

Early clinical trials testing the CD19-specific BiTE for patients with non-Hodgkin's B cell lymphoma achieved complete responses in patients with doses as low as 15 μg/m^2/day over 1 week intervals. Previously, several murine studies had demonstrated the potency of these constructs, which reported complete suppression of growth in subcutaneous tumor models at doses in the range of 1-10 μg/mouse/day over 5 days (De Jonge et al., 1998, Kriangkum et al., 2001, Wuest et al., 2001, Flieger et al., 2000). Because we observed comparable responses to EGFRvIII BiTE in our preclinical models of intracranial tumor at doses approximately 10-fold greater than those observed in corresponding preclinical experiments for previous constructs, we estimate that therapeutically relevant human doses of the EGFRvIII BiTE will begin at approximately

150 μg/m^2/day, which is roughly equivalent to 4 μg/kg/day for the average 60 kg adult (Reagan-Shaw et al., 2008). By stark contrast, recommended therapeutic doses of currently approved antitumor monoclonal antibodies range from an equivalent of 300 μg/kg/day (Herceptin) (Slamon et al., 2001) to up to 700 μg/kg/day (Avastin) (Hurwitz et al., 2004), further highlighting the exquisite potency of the BiTE therapeutic platform.

Given our encouraging results in preclinical models to date, our plan will be to pursue early human studies to determine the pharmacokinetic behavior of BiTEs and BiTE-activated T cells in a realistic clinical scenario. Using the microdosing approach, we will apply positron emission tomography (PET) and single photon emission computerized tomography (SPECT-CT) imaging to assess biodistribution of ^{111}In-labeled T lymphocytes and ^{124}I-radiolabeled EGFRvIII BiTE in patients with recurrent, biopsy-proven, EGFRvIII-positive GBM. Although CNS access will certainly be limited to some degree, the relative potency of the BiTE platform at extremely low doses (Bargou et al., 2008) suggests that only small amounts may actually need to reach the tumor in order to mediate significant therapeutic effects. Importantly, human microdosing studies will provide a permissive regulatory environment that will allow an early assessment of potential toxicity and efficacy of this BiTE at low doses that can be feasibly manufactured in an academic environment.

7.2.1 Preparation of Clinical EGFRvIII BiTE Reagent to Enable Human Microdosing Studies

Importantly, I have had the opportunity to work in a laboratory environment with a long history in the preparation, submission, and maintenance of investigator-held INDs, especially for monoclonal antibodies and immunoconjugates. It would otherwise be unfeasible to propose the production of recombinant antibodies in large quantities necessary for a microdosing trial. According to the FDA, Good Laboratory Practices (GLP) quality and procedures are acceptable for microdosed therapies, which are defined by a dose of no more than 30 nanomoles for protein products (equivalent to 150 μg). General preclinical toxicity studies are required, which consist of extended single dose toxicity in one species by intended route of administration, with accompanying hematology, clinical chemistry, necropsy, and histopathological data. Briefly, cDNA encoding the BiTE will be cloned into an expression vector under the T7-promoter and expressed in *E. coli.* Protein is prepared from inclusion bodies by standard method and purified using metal affinity chromatography as outlined in Chapter 3. In preparation for clinical studies, we will first prepare a master cell bank (MCB) expressing EGFRvIII BiTE. Although GLP quality procedures are sufficient for microdosing studies, we plan to adhere to more stringent Quality Control (QC)/Quality Assurance (QA) procedures such as (1) testing the MCB to ensure the following: identity of cells, plasmid purity, and safety; (2) developing the fermentation in a new 14L New Brunswick BioFlo 300 instrument; (3) recovering the expressed recombinant protein by centrifugation and

inclusion body preparation; (4) performing solubilization, reduction, and refolding in buffer (100 mM Tris pH 8.0, 2 mM EDTA, 0.5 M arginine hydrochloride, 0.9 mM oxidized glutathione, pH 10.2); (5) carrying out intermediate state preparation by concentration, diafiltration, and ammonium sulfate precipitation; and (6) conducting the purification process using metal affinity chromatography. Purified protein will be characterized for identity and impurity profiles, and the toxicology lot will then be produced, filled, and vialed. To remain in compliance with FDA standards for clinical-grade products, QC measures are in place either through in-house performance of required assays or by contract with certified outside agencies and service providers. These procedures should be in accordance with recommended guidelines and are the basis for an IND application. We will need to perform all efficacy, toxicity, potency, sterility, and stability studies described as follows. Potency study: cytotoxicity of EGFRvIII BiTE on cultured cell lines will be assayed by inhibition of protein synthesis as described previously. The biological activity of EGFRvIII BiTE will be defined by the degree of specific lysis against EGFRvIII-expressing tumor at a set E:T ratio of 20:1 and BiTE concentration of 10 μg/mL. Sterility study: quality control will be maintained by performance of, or contract for, all assays for bacterial, fungal, viral, pyrogen, and DNA contamination. Purified proteins are passed over an ActiCiean Etox resin (Sterogene Inc.) to remove any traces of endotoxins, which may be more prevalent in bacterially produced recombinant proteins. Standard sterility and limulus amebocyte lysate (LAL)

pyrogen tests are performed in the Department of Nuclear Medicine at Duke. These testing sites all meet FDA-required standards for such assays. Stability study: we will perform the following tests for the required stability verification: (1) Appearance, (2) pH, (3) A280, (4) SDS PAGE (Reduced and Non-reduced for both coomassie and silver staining), (5) SEC-HPLC (90% in the non-aggregated peak) (6) Endotoxin, (less than 5 endotoxin units per kg) (7) IEF, (8 to 9) (8) Sterility (bacterial, fungal and viral load as per USP797) (9) Container Closure, (10) Osmolality, (11) Potency and stability at 37°C and -80°. A Certificate of Analysis (COA) will be issued for each time point. Statistical analysis: the clinical BiTE reagent will be assessed and quality assurance will be carried out for biological activity as a measure of specific lysis *in vitro*. Previously validated batches have been analyzed in triplicate and a lysis rate calculated. Among these batches the mean (SD) lysis rate was 41.1% (3.6%). Based upon methods of statistical process control, future batches must achieve a specific lysis of 41.1% ± 2 * 3.6% at a set E:T ratio of 20:1 and BiTE concentration of 10 μg/mL. Any batch outside of these limits would not be acceptable.

7.2.2 Fully Human EGFRvIII-targeted BiTEs for Antitumor Therapy

Full murine antibodies and antibody fragments have been shown to serve as suitable platforms for therapy in clinical trials. However, it is also known that innate properties of murine-derived antibodies can lead to the development of neutralizing antibody responses (*i.e.*, human anti-murine antibody, or HAMA responses) that can

ultimately interfere with the ability for certain therapies to maintain efficacy over time (Tjandra et al., 1990). As such, using the same methodology as described in the design of the murine-derived EGFRvIII BiTE, we have generated cDNA encoding a fully human EGFRvIII-specific BiTE, designated 139x28F11. The antibody clones 139 (US 2010/0111979 A1) and 28F11 are fully human antibodies with specificity for the EGFRvIII tumor antigen and the human CD3 complex, respectively. 28F11 has been described as the fully human analog for the murine OKT3 clone, and was selected in this approach for its ability to bind CD3 and induce T cell activation through this receptor similarly to OKT3. This fully human EGFRvIII BiTE is currently in development, and will embody the ideal molecule for further development after it is appropriately validated. However, the current murine construct could certainly be translated in human studies without this effort, since several murine-based scFv products are being used without significant adverse consequences in clinical trials.

Table 9: cDNA sequence and design for a fully-human EGFRvIII-specific BiTE based on antibody clones 139 and 28F11.

139 V_H	GAGGTGCAGGTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGG GGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGCA GCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGG AGTGGGTCTCGGCTATTAGTGGTAGTGGTGGTAGTACAAACTACGC AGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAA GAACACACTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACA CGGCCGTCTATTACTGTGCTGGGAGCAGTGGCTGGTCCGAGTACTG GGGCCAGGGAACCCTGGTCACCGTCTCCTCG
139 V_L	GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAG GAGACAGAGTCACCATCACTTGCCGGGCTAGTCAGGGCATTAGAA ATAATTTAGCCTGGTATCAGCAGAAACCAGGGAAAGCCCCTAAGC GCCTGATCTATGCTGCCTCCAATTTGCAAAGTGGGGTCCCATCAAG GTTCACCGGCAGTGGATCTGGGACAGAATTCACTCTCATAGTCAGC AGCCTGCAGCCTGAAGATTTTGCGACTTATTACTGTCTACAGCATCA CAGTTACCCGCTCACTTCCGGCGGAGGGACCAAGGTGGAGATCAAA
Gly$_4$Ser	GGTGGTGGCGGTTCA
28F11 V_H	CAGGTGCAGCTGGTGGAGTCCGGGGGAGGCGTGGTCCAGCCTGGG AGGTCCCTGAGACTCTCCTGTGCAGCGTCTGGATTCAAGTTCAGTG GCTATGGCATGCACTGGGTCCGCCAGGCTCCAGGCAAGGGGCTGG AGTGGGTGGCAGTTATATGGTATGATGGAAGTAAGAAATACTATG TAGACTCCGTGAAGGGCCGCTTCACCATCTCCAGAGACAATTCCA AGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGAC ACGGCTGTGTATTACTGTGCGAGACAAATGGGCTACTGGCACTTC GATCTCTGGGGCCGTGGCACCCTGGTCACTGTCTCCTCA
28F11 V_L	GAAATTGTGTTGACACAGTCTCCAGCCACCCTGTCTTTGTCTCCAGG GGAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCAGC TACTTAGCCTGGTACCAACAGAAACCTGGCCAGGCTCCCAGGCTCCT CATCTATGATGCATCCAACAGGGCCACTGGCATCCCAGCCAGGTTCA GTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGCCTA GAGCCTGAAGATTTTGCAGTTTATTACTGTCAGCAGCGTAGCAACTG GCCTCCGCTCACTT TCGGCGGAGGGACCAAGGTGGAGATCAAA

The 15-mer, glycine-serine linkers typically used to bridge variable light and heavy domains within single-chain antibodies is omitted for simplicity.

7.2.3 Microdosing Objectives and Study Design

Based on calculations from preclinical data, we would plan to administer the EGFRvIII BiTE to patients at a minimal therapeutic dose of 150 μg/m^2, which is also the maximum dose allowable under regulatory restrictions for microdosing studies. In collaboration with Dr. Michael Zalutsky, we have extensive experience radiolabeling antibody-based constructs for animal localization studies and human use (Zalutsky et al., 1996, Scott et al., 2007b). We will radiolabel these constructs and inject them intravenously together with ^{111}In-labeled autologous T cells as outlined in detail below. Subjects included in our study will be limited to patients with recurrent, biopsy-proven EGFRvIII expression on >10% of their cells based on IHC from biopsy or resection. Patient population: Our proposed Phase 0/1 microdosing study will enroll a unique set of adults with recurrent supratentorial EGFRvIII$^+$ GBM who have undergone surgical biopsy or excision with residual disease (>1 cm), have a Karnofsky performance status ≥ 70, have stable or decreasing doses of steroids, have no other serious illnesses, and have normal OP4 and CMP. Treatment plan: upon enrollment, patients will be randomized to two groups, A or B, in a crossover**,** repeated measures study design. Group A will receive a sequence of two treatments, first ^{111}In-labeled T cells alone, followed by ^{111}In-labeled T cells with radiolabeled EGFRvIII BiTE. Group B will receive the treatments in the reverse order. Treatments will be separated by 7 days, allowing for sufficient clearance of the BiTE, which has a half-life of approximately 2 hours. Following each

treatment, blood samples will be drawn at 1 and 6 hours, then 1, 2, and 3 days after start of treatment. Daily imaging by PET and SPECT (days 1, 3, and 5) will be used to determine the intracerebral localization of ^{111}In-labeled T cells and iodinated BiTE. Quantification of radioactivity will be assessed by previously published methods that calculate counts from reconstructed images based on the percentage of injected dose. Lymphocytes in patients' peripheral blood will be analyzed by FACS and quantified. Isolated PBMC will be stained with fluorescence-labeled antibodies against cell surface or intracellular markers including TCR, CD3, CD4, CD8, CD56 and the activation marker CD69. Monitoring plan: patients with recurrent EGFRvIII-positive GBM will be imaged by MRI for baseline measurements and to assess progression prior to being randomized to receive EGFRvIII BiTE; although the purpose of this study is not to detect tumor responses, any evidence of tumor response will be determined by serial measures of the product of the 2 largest cross-sectional diameters (Macdonald et al., 1990, Galanis et al., 2006). For patients with < 0.5mm of enhancing tissue in 2 cross-sectional diameters in each of 2 perpendicular slices on the baseline scan, progression will be defined when enhancement is $\geq$ 1 cm in 2 cross-sectional diameters in each of 2 perpendicular slices. The modified RANO criteria (Wen et al., 2010) will be used for overall assessment of tumor response. Tumor progression will be documented histologically, unless there are clinical contraindications, to exclude inflammatory responses presenting as radiographic or clinical changes, which could indicate potentially toxic or therapeutic responses and

not tumor progression. If tissue is obtained, it will be used to confirm tumor progression histologically and to assess immunologic cell infiltration and antigen escape using IHC. Overall survival will be defined as time between enrollment and death, and will be censored at the last follow-up. Safety monitoring: a dose limiting toxicity (DLT) will be defined as any drug-related Grade IV or non-neurologic Grade III toxicity of any duration. A Grade III neurologic toxicity will only be declared a DLT if not reversible within 4 weeks.

7.3 Caveats for Future Clinical Trials in Brain Tumor Immunotherapy

One challenge in the translation of antitumor immunotherapy has been the absence of a methodological framework specifically designed for early phase clinical studies of immune-based treatments. Interestingly, clinical trials in immunotherapy have historically followed paradigms similar to those that have been established for chemotherapy. This however is likely inappropriate given the vast dissimilarities between the two forms of treatment with regard to their respective mechanisms-of-action and other pharmacodynamic properties. For instance, unlike chemotherapy, immune-based antitumor vaccines often do not have an identifiable maximum tolerated dose (MTD); instead they frequently seek to identify a maximum *feasible* dose, based on the amount of "drug" that can be manufactured from limited biological material. Moreover, for most immunotherapies, the administered dose has not been ostensibly linked to endpoints including clinical efficacy or the magnitude of antitumor immune

responses. Lastly, again in contrast to many other types of treatment, it has been suggested that favorable clinical outcomes following immunologic responses are delayed and in some cases may occur even after radiographic evidence of tumor progression, drawing further attention to whether current endpoint criteria for assessing the impact of immune-based treatments might be adjusted to better detect significant responses (Wolchok et al., 2009).

Along those lines, a notable observation in immunotherapy trials has been the seemingly incongruous finding in some patients of unaltered time to progression, yet improved overall survival. Because this scenario is considered unusual for chemotherapies and other directly cytotoxic therapies, WHO or RECIST criteria (Eisenhauer et al., 2009) have traditionally mandated such outcomes should be labeled progressive disease (PD), which, due to certain conventions has become synonymous with drug failure. However, in the setting of immune therapy in particular, this approach may be suboptimal, not only because it has the potential to dilute observed therapeutic effects but also to completely cease experimental therapy. As a result, groups such as the Cancer Immunotherapy Consortium of the Cancer Research Institute have attempted to design more appropriate "immune-response criteria" to be applied during immunotherapy trials (Hoos et al., 2010, O'Regan et al., 2011, Wolchok et al., 2009). Importantly, these new criteria broaden the types of observed patterns in tumor growth that might qualify a response to therapy, in order to foster more accurate

alignment between clinical outcome (*i.e.*, overall survival) and radiographic metrics of tumor progression. While not yet universally accepted, these criteria continue to expand in recognition and use.

7.3.1 Considerations for Immune Monitoring

Owing to the inherent variability associated with survival endpoints, clinical studies of immunotherapy for glioma have historically focused on measures of immunity as surrogates for objective clinical response. Several immunological assays have been employed for this purpose including delayed type hypersensitivity (DTH) reactions, enzyme-linked immunosorbent spot (ELISpot), tetramer analysis, lymphoproliferative assays, intracellular cytokine staining (ICS), and an array of *in vitro* cytotoxicity assays thought to be predictive of antitumor efficacy *in vivo*. While they have certainly provided valuable insight, none of these metrics have been universally validated to date, largely due to their inability to consistently correlate with clinical outcomes. Numerous efforts are underway to establish alternative markers for antitumor immunotherapy; however, until these parameters are fully validated, care should be taken to avoid over-interpretation of immunologic surrogate data.

It is reasonable to speculate that given current shortcomings, immune monitoring will likely transition from a predominant focus on T-cell responses to a broader, more global view of host immunity. For instance, increasing attention may be placed on previously neglected immune cell types including monocytes, natural killer

cells, and other effectors that may favorably contribute to antitumor immunity. Moreover, efforts to assess the immunological status of the brain tumor microenvironment—as opposed to analyses limited to cells present in peripheral blood—may also reveal predictive data that have been underappreciated to date.

Importantly, it should be noted that past failures in immune monitoring may be as much of a result of technical limitations as they are of incomplete scientific understanding. That is to say, currently available immune assays (*e.g.*, ELISpot, ICS, etc.) have been shown to suffer from significant methodological inconsistencies between laboratories, leading to a state of generalized variability and unreliability in the interpretation of immune response data (Hoos et al., 2011). To address this issue, recent studies have developed tools for assay harmonization that would implement widespread adoption of standard operating procedures; such efforts have been shown to significantly mitigate several of these inconsistencies (Britten et al., 2009, Hoos et al., 2010) and should thus be strongly considered for integration into future plans for clinical development.

7.4 Conclusion

Given recent progress toward approval for other tumors, the use of immunotherapy to treat malignant glioma may provide a promising option for future study, where the need for safer, more effective treatment is great. In order to continue to advance immune-based treatments, such as BiTEs, for patients with brain tumors, it

will be necessary to broadly address a number of limitations that pertain especially to the treatment of tumors in the CNS. Among these include aspects of drug delivery, glioma-induced immune suppression, and methodological inconsistencies in clinical trial design. Furthermore, special consideration will have to be dedicated to the use of vaccines and other immune-based treatment in the context of standard-of-care treatments including radiation and chemotherapy. Successful treatment of these devastating CNS tumors will likely require a combinatorial, multi-pronged approach, and further studies will seek to elucidate the ways in which immune-based therapy might complement or even work synergistically with these existing strategies.

Appendix

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Biography

Bryan Daehahn Choi was born in Cleveland, Ohio, on September 20, 1983, the son of Miehyun Claire Choi (née Riew) and Peter Yongwoo Choi. He attended Revere High School in Richfield, Ohio, and served as both student body president and valedictorian in 2002. He then attended Harvard College, where he was elected to *Phi Beta Kappa* and completed an A.B. *summa cum laude* in three years, supported by an undergraduate award from the National Science Foundation. Choi matriculated at Duke University School of Medicine in 2006, where he was elected president of the *Alpha Omega Alpha* medical honor society. At Duke University, Choi pursued dual medical and graduate degree training under the mentorship of esteemed neurosurgeon-scientist John H. Sampson and internationally renowned brain tumor expert, Darell D. Bigner.

During his doctoral training at Duke University, Choi published in numerous scientific books and journals including manuscripts in *Proceedings of the National Academy of Sciences of the United States of America*, *Nature Chemical Biology*, *Clinical Cancer Research*, *Brain Pathology*, and *Oncoimmunology*, among several others. His work has been recognized through many NIH and private foundations grants, including the prestigious Cancer Research Institute STaRT Grant and an NIH/NCI F30 National Research Service Award. In recognition of his commitment to enhance the quality of care for patients with brain tumors, Choi was awarded an Albert Schweitzer Fellowship in 2011. Choi anticipates a clinical and research career in the field of neurosurgery.